Original Article LncRNA CRNDE promotes hepatocellular carcinoma cell proliferation and upregulates cyclin D1 expression

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Abstract: Hepatocellular carcinoma (HCC) is a major cause of cancer-related deaths. CRNDE is the most highly expressed IncRNA in HCC, suggesting that CRNDE may be involved in the development and biological behavior of HCC. However, the underlying mechanisms by which CRNDE promotes cell proliferation are still largely unclear. This present study reports that CRNDE was upregulated in HCC tissues and cells. *In situ* hybridization assay showed that CRNDE specific staining was observed in the nucleus of SMMC7721 and HepG2 cells. Furthermore, overexpression of CRNDE significantly enhanced cell proliferation and upregulated cyclin D1 expression, whereas short hairpin RNA knockdown of CRNDE caused inhibition of cell proliferation and downregulation of cyclin D1. CRNDE may serve as a candidate prognostic biomarker and target for new therapies targeting human HCC.

Keywords: Hepatocellular carcinoma, CRNDE, cell proliferation, cyclin D1

Introduction

Hepatocellular carcinoma (HCC) is a major cause of cancer-related deaths. Numerous studies have reported that long non-coding RNA (IncRNA) colorectal neoplasia differentially expressed (CRNDE) is activated early in colorectal neoplasia. It is also upregulated in many other solid tumors. CRNDE is overexpressed in a variety of other tumor cells such as lung adenocarcinoma and gastric cancer cells [1-4]. CRNDE is the most highly expressed IncRNA in HCC, suggesting that CRNDE may be involved in the development and biological behavior of HCC [5-7]. However, the underlying mechanisms by which CRNDE promotes cell proliferation are still largely unclear.

This study investigated expression and the role of CRNDE in HCC. It was demonstrated that CRNDE levels were remarkably upregulated in HCC tissues. CRNDE promoted HCC cell proliferation. Additionally, overexpression of CRNDE induced cell cycle progression by accelerating G1/S and G2/M transition concomitantly with upregulation of cyclin D1.

Materials and methods

HCC tissue samples and cells culture

Ten pairs of tumor and adjacent non-tumor tissues were collected from surgical resections of HCC in the Affiliated Hospital of Guizhou Medical University. Specimens were snap-frozen in liquid nitrogen and stored at -80°C. All patients received surgery, without preoperative chemotherapy or radiation therapy. All patients provided written informed consent and ethical consent was granted from Committees for Ethical Review of Research of the Affiliated Hospital of Guizhou Medical University (Guizhou, China).

Human hepatocellular carcinoma (HCC) cell lines (SMMC7721, SK-hep1, Huh7 and HepG2), human immortalized normal human liver cell line (LO2), and embryonic kidney cell line (293T) were obtained from the Chinese Academy of Sciences Cell Bank. They were cultured in Dulbecco's Modified Eagle Medium (DMEM) of high glucose with 10% fetal bovine serum (FBS, BI, ISR). All cells were incubated at 37°C in a humidified atmosphere with 5% CO₂.

RNA extraction and qRT-PCR

Total RNA was isolated with TRIzol Reagent (Invitrogen, Carlsbad, CA) and treated with RNase-free DNase (Promega, USA). For CRN-DE, first-strand cDNA was generated using PrimeScript RT reagent kit with gDNA Eraser, according to manufacturer instructions (Takara). Primers used were 5'-ATATTCAG CCGT-TGGTCTTTGA-3' (forward) and 5'-TCTGCGTGA-CAACTGAG GAT TT-3' (reverse). GAPDH was used for normalization and the primers were 5'-CGACCACTTTGTCAAGCTCA-3' (for-ward), 5'-AGGGGTCTACATGGCAAC TG-3' (reverse). The other forward and reverse primers of gRT-PCR were as follows: cyclin D1-F: 5'-GTTCGT-GGCCTCTAAGATGAAG-3', cyclin D1-R: 5'-GATG-GAGTTGTCGGTGTAGATG-3'. All quantitative real-time polymerase chain reaction (gRT-PCR) samples were performed using UltraSYBR mixture (Cwbio, China) and conducted using CFX Connect TM real-time PCR system (Bio-Rad). Quantification analysis was analyzed by the $2^{-\Delta\Delta CT}$ method.

In situ hybridization

In situ hybridization was used for detection in cell lines with probes targeting CRNDE, following manufacturer protocol. Signals were detected using Fluorescein (FITC)-conjugated Affinipure Goat Anti-Rabbit IgG(H+L) (SA00-003-2, Proteintech, Wuhan, China) at 37°C for 30 minutes. Nuclei were counterstained with DAPI. Immunofluorescence was then detected under FV1000 fluorescence microscope (Olympus, Tokyo, Japan).

Western blot analysis

Cells were lysed in RIPA Buffer (Beyotime, China) and supplemented with 1 mmol/L PMSF. Protein concentration was measured by BCAAassay Kit (Beyotime, China). Proteins were separated on a 10-12% SDS-PAGE gel and then transferred to PVDF membranes. Membranes were blocked with 5% milk, incubated overnight at 4°C with a primary rabbit antibody against cyclin D1 (1:500 dilution, Bioword), and washed three times in TBST. Blots were then incubated with a goat anti-rabbit or anti-mouse HRP secondary antibody (Bioword, USA, 1:1000 dilution). Finally, ECL Detection Reagent (Millipore, Billerica, MA) was used for signal detection. Data were normalized to GAPDH (1:2000, Pro-teintech).

Plasmids, short-hairpin (sh) RNA, and transfection

pcDNA3.1-CRNDE was purchased from Gene-Pharma (Shanghai, China). Short-hairpin RNA targeting human CRNDE were ligated into the pGreenPuro shRNA vector (SBI, USA) according to manufacturer protocol. Transfections were performed with a Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA), according to manufacturer instructions. Cells were harvested 48-72 hours after transfection.

MTS assay

Cells (2000 cells/well) were seeded into 96 well plates, after 24 hours of transfection, and measured at different time points (0, 24, 48 and 72 hours) using the MTS kit (Cell Titer 96 ® Aqueous One Solution Cell Proliferation Assay, Promega, USA), followed manufacturer protocol. The 490 nm wave-length absorption value was measured. All experiments were performed in triplicate and repeated 3 times.

Colony formation assay

Cells (1 × 10^4 cells/well) were seeded into 6well plates, after 24 hours of transfection, and cultured for 4 days, while 4% paraformaldehyde was used to fix with cells. Cells were then stained with a Crystal Violet cell colony staining kit (GenMed Scientifics), according to manufacturer instructions.

Cell cycle analysis

Cells were harvested and washed twice with PBS. After bein fixed in 70% ice-cold ethanol and kept overnight at 4°C, cells were stained with propidium iodide supplemented with RNaseA (Keygen Biotech) for 30 minutes at 37°C. DNA content of labeled cells was acquired using FACS cytometry (BD Biosciences Inc., Franklin Lakes, NJ, USA). Each experiment was performed in triplicate.

Immunohistochemistry

Immunohistochemistry for Ki-67 was performed on paraffin sections using a primary antibody against Ki67 (Pro-teintech) and horserad-



Figure 1. CRNDE significantly upregulated in HCC tissues and cell lines. A. CRNDE expression level was examined in 10 paired HCC tissues (T) and non-tumor tissues (N) by qRT-PCR. Transcript levels were normalized to GAPDH expression. B. CRNDE expression level was examined in HCC cell lines (SMMC7721, Huh7, SK-hep1 and HepG2) and one normal liver cell line LO2 by qRT-PCR. Transcript levels were normalized to GAPDH expression. C, D. The tumor sections were under IHC staining using antibodies against ki-67, representative graphs are shown in #1 sample. **P < 0.01. E. RNA ISH assay of CRNDE in HCC cells. The green signal is from the FITC-labeled RNA probe. F. CRNDE expression level was examined after cells were transfected with pcDNA3.1, pcDNA3.1-CRNDE, sh-NC, and sh-CRNDE at 48 hours in SMCC7721 and HepG2 cells. Transcript levels were normalized to GAPDH expression. **P < 0.01.

ish peroxidase-conjugated rabbit anti-goat antibody (Maixin, Fuzhou, China). Proteins *in situ* were visualized with 3, 3-diaminobenzidine and analyzed using a bright field microscope.

Statistical analysis

SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad software (GraphPad Software, Inc., La Jolla, CA, USA) were used to analyze all data for statistical significance. Two-tailed Student's t-test was used for comparisons of two independent groups. Statistical si-

gnificance was set at *P < 0.05, **P < 0.01. P < 0.05 was considered statistically significant.

Results

CRNDE and Ki67 significantly upregulated in HCC

Expression of CRNDE, in 10 paired HCC tissues (T) and non-tumor tissues (N), was examined by quantitative real time RT-PCR (qRT-PCR). It was found that CRNDE expression was higher in 10 paired HCC tissues than non-tumor tissues



Figure 2. CRNDE promoted cell proliferation in HCC. A, B. MTS cell proliferation was detected after cells were transfected with pcDNA3.1, pcDNA3.1-CRNDE, sh-NC, and sh-CRNDE at 0, 24, 48 and 72 hours in SMCC7721 and HepG2 cells. Representative graphs are shown. C. Cell colonies were calculated after cells were transfected with pcDNA3.1, pcDNA3.1-CRNDE, sh-NC and sh-CRNDE at 14 days in SMCC7721 and HepG2 cells. Representative graphs are shown. The data graphs depict the count number from three independent experiments.

(Figure 1A). Furthermore, the results indicated that CRNDE was obviously upregulated in HCC cell lines (SMMC7721, Huh7, SK-hep1 and HepG2) compared with that in normal liver cell line L02 (Figure 1B).

Immunohistochemistry analysis of the tumor tissues revealed that expression of Ki67 proliferation antigen was significantly stronger in tissues (T) than non-tumor tissues (N) (**Figure 1C**, **1D**). Next, *in situ* hybridization assay was performed on HCC cells with results showing that CRNDE specific staining was observed in the nucleus of SMMC7721 and HepG2 cells (**Figure 1E**). SMMC7721 and HepG2 cells were selected for functional experiments. First, the effect of overexpression of CRNDE (pcDNA3.1CRNDE) and knockdown of CRNDE (sh-CRNDE) were assessed. It was found that CRNDE was drastically increased after transfection with pcDNA3.1-CRNDE plasmid and obviously decreased after transfection with sh-CRNDE plasmid for 48 hours (Figure 1F).

CRNDE promotes cell proliferation in HCC

To determine proliferation of CRNDE in HCC cells, a pcDNA3.1-CRNDE plasmid that could overexpress CRNDE and knockdown of CRNDE (sh-CRNDE) was constructed. MTS assay results verified that overexpression of CRNDE significantly increased cell proliferation and knockdown of CRNDE dramatically suppressed cell proliferation rate, compared with con-

CRNDE was upregulated in HCC CRNDE promotes proliferation and upregulates cyclin D1



Figure 3. CRNDE induces cell-cycle progression in HCC cells. A. Cell-cycle analysis of HepG2 and SMMC-7721 cells overexpressing CRNDE and stably silenced CRNDE expression. B. Proportion of cells in various phases of the cell cycle. **P < 0.01, *P < 0.05.



Figure 4. CRNDE upregulated cyclin D1 expression in HCC cells. A. qRT-PCR analysis of cyclin D1 mRNA expression when SMMC7721 and HepG2 cells were transfected with pcDNA3.1-CRNDE or sh-CRNDE. Transcript levels were normalized to GAPDH expression. **P < 0.01, *P < 0.05. B, C. Western blot analysis of CRNDE regulated expression of cyclin D1, using GAPDH as endogenous control. **P < 0.01.

trol group, at 48 and 72 hours in SMMC7721 and HepG2 cells. (Figure 2A, 2B). Colony formation assay showed overexpression of CR-NDE displayed more clones while the clone number significantly decreased after knockdown of CRNDE, compared with control groups (Figure 2C).

CRNDE induces cell-cycle progression in HCC cells

To gain insight into mechanisms by which CRNDE enhances HCC cell proliferation, fluorescence-activated cell sorting (FACS) was performed to analyze differences in cell-cycle distribution after CRNDE overexpression or silencing. The results revealed a reduction in G1 population and an increase in the S and G2/M population in HepG2 and SMMC-7721 cells overexpressing CRNDE. Conversely, repressed CRNDE expression mainly led to G1 accumulation and a decrease of S and G2/M phase (Figure 3A, 3B).

CRNDE upregulates cyclin D1 expression in HCC cells

This study, next, examined levels of cell cycle related cyclin D1 genes in SMMC7721 and HepG2 cells by stably overexpressing CRNDE and silencing CRNDE expression, through qRT-PCR and Western blot analysis. Overexpression of CRNDE elevated expression of cyclin D1. In contrast, knockdown of CRNDE resulted in decreased expression of cyclin D1 (Figure 4A-C).

Discussion

HCC is one of the most common cancers wor-Idwide with high prevalence and lethality [8]. Multiple evidences have illustrated that aberrant IncRNA expression is engaged in various cancers. Targeted therapies, applying IncRNA as a novel diagnostic and therapeutic tool, have obtained more and more attention, including MALAT1 [9], H19 [10], and HANR [11]. CRNDE, a long-noncoding RNA, promotes glioma cell growth and invasion through mTOR signaling [12]. Highly expressed IncRNA CRNDE promotes cell proliferation, through Wnt/betacatenin signaling, in renal cell carcinoma [4]. Long noncoding RNA CRNDE promotes multiple myeloma cell growth by suppressing miR-451 [3]. Long noncoding RNA CRNDE, stabilized by hnRNPUL2, has accelerated cell proliferation and migration in colorectal carcinoma via activating Ras/MAPK signaling pathways [13]. Long noncoding RNA CRNDE activates Wnt/betacatenin signaling pathways through acting as a molecular sponge of microRNA-136 in human breast cancer [14]. LncRNA CRNDE promotes colorectal cancer cell proliferation and chemoresistance via miR-181a-5p-mediated regulation of Wnt/beta-catenin signaling [15]. This present study found that CRNDE was remarkably increased in HCC tissues. Overexpression of CRNDE promoted, while knockdown of CRNDE suppressed, HCC cell proliferation *in vitro*, consistent with previous studies.

CIAPIN1 inhibits gastric cancer cell proliferation and cell cycle progression by downregulating cyclin D1 and upregulating P27 [16]. Upregulation of cyclin D1 and Bcl2A1 by insulin is involved in osteoclast proliferation [17]. Overexpression of CARM1 promotes human osteosarcoma cell proliferation through pGSK3beta/ beta-Catenin/cyclin D1 signaling pathways [18]. Cyclin D1 belongs to the cyclin protein family and has three subtypes: D1, D2 and D3. Among them, D1 has been strongly associated with cancer. Cyclin D1 is dominant at G0/G1 phase and is synthesized at G1 phase. It, then, forms cyclin D1-CDK4 or cyclin D1-CDK6 complexes, activates CDK, and phosphorylates the key substrate retinoblastoma gene (Rb), enabling cells to enter S phase through the restriction point. Upregulated expression of cyclin D1 promotes phosphorylation of pRb and accelerates entry into S phase, resulting in promoted cell proliferation [19]. To the best of our knowledge, this current research is the first to offer evidence for a novel mechanistic correlation between CRNDE and cyclin D1 in HCC. Moreover, this study demonstrates that overexpression of CRNDE upregulates expression of cyclin D1 while knockdown of CRNDE suppresses expression of cyclin D1.

In summary, these findings show that IncRNA CRNDE was upregulated in HCC tissues and cells. Promotion of CRNDE on HCC cell proliferation and tumorigenesis may partly be through epigenetically inducing cyclin D1. These results reveal that CRNDE functions as one of the oncogenes in HCC and might be regarded as a critical target and therapeutic tool for HCC in clinical application. However, other possible mechanisms by which CRNDE participates in the biological function of HCC cells are not fully understood.

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Disclosure of conflict of interest

None.

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