Original Article YY1-mediated reticulocalbin-2 upregulation promotes the hepatocellular carcinoma progression via activating MYC signaling

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Abstract: Hepatocellular carcinoma (HCC) is a common digestive tumor with high fatality worldwide. Previous studies have shown that Reticulocalbin-2 (RCN2) was a crucial factor for HCC proliferation, but invasion and migration mechanism of RCN2 contributing to HCC is poorly investigated. In this study, we estimated the RCN2 expression in both patient tissues and cell lines by polymerase chain reaction (PCR) and western blotting (WB), as well as the clinical information of HCC patients from public databases. Biological function induced by RCN2 in *vitro* and *vivo* was also researched through multiple functional experiments. Upstream and downstream signal of RCN2 was identified by bioinformatics. We found that up-regulated RCN2 was related to poorer prognosis in HCC patients and attached significance to HCC proliferation, invasion and migration. Luciferase reporter assay and chromatin immunoprecipitation validated that YY1 as the upstream transcription factor of RCN2, facilitating the expression of RCN2. Gene set enrichment analysis indicated that HCC progression induced by RCN2 might be related to MYC signaling. Furthermore, we demonstrated RCN2 reduced proteasomal degradation of MYC and lead to HCC progression. The effects of overexpressed RCN2 in HCC were attenuated by MYC silencing. In conclusion, our study highlighted the vital role of RCN2 in tumor progression and the potential benefit for the treatment of HCC.

Keywords: Hepatocellular carcinoma, reticulocalbin-2 (RCN2), YY1, MYC

Introduction

Hepatocellular carcinoma (HCC), as the most crucial primary liver cancer, is the sixth most frequent cancer worldwide and the leading cause of cancer mortality [1, 2]. Patients with HCC usually have a poor prognosis and the estimated 5-year survival rate is 21% in USA and 10.1% in China [3, 4]. Furthermore, both morbidity and mortality rates for HCC are increasing in recent years [5, 6]. It's common cases for local recurrence and distant metastasis in HCC, because of lacking diagnostic markers to assess the HCC progression. Therefore, searching for a valid prognostic biomarker for metastatic HCC is a momentous goal.

Reticulocalbin-2 (RCN2), also named E6-binding protein (E6BP) or endoplasmic reticulum Ca^{2+} -binding protein of 55 kDa (ERC55), is strictly

located in endoplasmic reticulum [7, 8], RCN2 is a low-affinity Ca²⁺ binding protein localized to the secretory pathway of mammalian cells, belongs to a member of the EF-hand Ca2+ binding protein family, which participate in the secretory process, chaperone activity, signal transduction as well as participation in a large variety of disease processes [9]. As for cancer research, several studies showed that RCN2 was related to tumor progression, such as mammary cancer, cervical cancer, neck squamous cell carcinoma and colorectal cancer [10-13]. Ding et al. [14] found that RCN2 played a pivotal role in HCC pathogenesis through regulating activation of the epidermal growth factor receptor (EGFR) pathway and modulating epidermal growth factor receptor EGFR dimerization and internalization. However, the roles of RCN2 in HCC invasion and migration remain unknow.

MYC, one of the most important mutated oncogenes, is overexpressed in both high-grade premalignancy and invasive tumors [15]. A growing body of researches revealed that MYC mediated cellular invasion and migration through regulating specific programs in HCC. Recent study showed that MYC binding with the intracellular chloride channel protein (CLIC1) and enhanced the transcription activity of MYC to downstream genes, promoted HCC cells proliferation, invasion and migration [16]. Another study suggested that mammalian target of rapamycin complex 2 (mTORC2) was activated in MYC-driven HCC, leading to phosphorylation/ activation of serine/threonine kinase 1, promoted hepatocarcinogenesis in mice and humans [17].

In this study, we validated that RCN2 mRNA and protein levels were overexpressed in HCC samples and were related to poorer tumor grade and cancer stage. Biological function study suggested that upregulated RCN2 in HCC cells promoted invasion and migration in *vitro* and *vivo*. Additionally, we demonstrated that YY1 transcription factor involved in the transcriptional regulation of RCN2, which promoted invasion and migration via MYC signaling in HCC.

Materials and methods

Clinical samples and cell culture

40 paired confirmed HCC tissues and adjacent non-tumor liver tissues were obtained from patients who underwent hepatectomy in Zhongnan Hospital of Wuhan University with informed consent. And this study was approved by the ethics committee of Zhongnan Hospital. Human HCC cell lines Huh7, HCCLM3, Hep3B, HepG2, SK-hep1 and immortalized human liver cell line HL-7702 (LO2) were procured from the cell bank of Type Culture Collection (Shanghai, China), and were cultured in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) at 37°C with 5% CO₂.

RNA isolation and quantitative real-time PCR analysis

Total RNA was isolated from the HCC tissues and cells with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). The reverse transcriptase of mRNA was extracted with PrimeScript RT reagent Kit (Takara, Tokyo, Japan). Quantitative real-time PCR was implemented with SYBR Green PCR kit (Toyobo, Osaka, Japan) on a Biorad-CFX96 thermal cycler (Hercules, USA). Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method. The primers involved were as follows: RCN2: forward (5'-TTCAGGTCCCGGTTTGAGTCT-3'), reverse (5'-TCAAGCCTGCCATCGTTATCT-3'); YY1: forward (5'-ACGGCTTCGAGGATCAGATTC-3'), reverse (5'-TGACCAGCGTTTGTTCAATGT-3'); MYC: forward (5'-GTCAAGAGGCGAACACAACA 3'), reverse (5'-TTGGACGGACAGGATGTATGC-3'); GAPDH: forward (5'-GAAGATGGTGAAGGTCGGAG-TC-3'), reverse (5'-GAAGATGGTGATGGGATTTC-3').

Lentiviral construction, RNA interference, and cell transfections

Lentiviral vectors carrying RCN2-cDNA and control plasmids were designed and synthesized by Genechem (Shanghai, China). MYC plasmids were obtained from Genecreate (Wuhan, China). HA-Ubiquitin plasmids were gifted from Dr Gaosong Wu's Research Group of the Department of Thyroid and Breast Surgery. Zhongnan Hospital of Wuhan University [18]. Small interfering RNAs (siRNAs) targeting RCN2, YY1 and MYC were purchased from Genecreate (Wuhan, China). Cells were transfected with 10 nM siRNA with Lipofectamine RNAiMAX Reagent (Thermo Fisher Scientific, Waltham, MA, USA). The RCN2-siRNA, YY1siRNA and MYC-siRNA sequences were as follows: siRCN2-1 (5'-GACGGAAUUUGUCAUUCA-ATT-3'), siRCN2-2 (5'-CUUGGGUAGUACCUAAU-AATT-3'), siYY1-1 (5'-GCCUCUCCUUUGUAUAU-UAUUTT-3'), siYY1-2 (GACGACGACUACAUUGA-ACAATT), siMYC-1 (5'-GAGGUAUCUGGAAGAAA-UTT-3'), siMYC-2 (5'-GGAACAAGAAGAUGAGGA-ATT-3') and siControl (5'-UUCUCCGAACGUGU-CACGU-3').

Immunohistochemistry (IHC)

Clinical tissues were fixed in 4% paraformaldehyde, embedded in paraffn and cut into sections with a thickness of 4 μ m. Then, the sections were deparaffnized, rehydrated and incubated in sodium citrate and 3% H₂O₂. Next, the samples were incubated in bovine serum albumin and the primary antibody overnight. The next day, the sections were incubated with horseradish peroxidase (HRP) conjugated secondary antibody. After that, the sections were

stained with hematoxylin and observed via microscope.

Cell proliferation assay

Cells transfected as indicated were plated in 96-well plates at a density of 5×10^3 cells/well cultured in DMEM with 10% FBS. Cell proliferation was evaluated with Cell Counting Kit-8 (CCK-8; Dojindo, Japan) assays following the manufacturer's protocol. And the absorbance at 450 nm was measured by microplate reader.

Transwell invasion assay

Transwell invasion assay was performed with 8 μ m pores (BD Biosciences, USA) and 24-well plates. A total of 2×10⁴ cells transfected as indicated were seeded in the upper chamber with serum-free medium and DMEM with 10% FBS was placed in the lower chamber. The invaded cells in the lower chamber were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet after incubating for 48 h. The number of invasion cells were counted via microscope.

Wound healing assay

Cells transfected as indicated were plated in 6-well plates at a density of 1×10^6 cells/well and cultured in DMEM without FBS. Wounds were scratched a line using a 200 µl pipette tip. The areas of scratches were measured after 0 h, 24 h or 48 h hours under a microscope.

Western blotting (WB)

Tissues and cells were treated with RIPA lysis buffer with protease inhibitors and phosphatase inhibitors (Roche, Germany). Same amounts of proteins were assessed with the BCA Protein Assay Kit (Thermo Scientific). Next, proteins were separated on 10% SDS-PAGE and were transferred to polyvinylidene fluoride membranes (Millipore, USA). Membranes were incubated successively with 5% skim milk and primary antibodies. After that, the membranes were incubated with HRP conjugated secondary antibody. Proteins were detected with ECL luminous fluid (Bio-Rad). The specific primary antibodies were as follow: RCN2 (1:1000, Proteintech), YY1 (1:5000, Proteintech), MYC (1:1000, Proteintech), N-Cadherin (1:1000, Abcam), E-Cadherin (1:1000, Abcam), Vimentin (1:500, Abcam), Ubiquitin (1:1000, Proteintech), HA-Tag (1:1000, Cell Signaling Technology), GAPDH (1:5000, Proteintech).

Dual luciferase reporter activity assay

Dual luciferase assay was performed as per standard protocols (Promega, Madison, USA). The luciferase gene was cloned downstream of the wild-type or mutant region of the RCN2, and co-transfected into Huh7 with the pcDNA3.1-YY1 and control vector. After 48 hours, the cells were lysed and the firefly luciferase activity was measured using the Dual-Luciferase® Reporter Assay System (Promega) normalized to Renilla luciferase as the internal reference.

Chromatin immunoprecipitation (ChIP) assay

Magna ChIP-seq[™] Chromatin Immunoprecipitation Kit (Millipore, Billerica, USA) was used for detecting protein-chromatin interactions. Briefly, the cells were fixed with formaldehyde and sonicated, and incubated with the target protein. The cross-linked DNA fragments were then released from the co-precipitated complexes, purified, and amplified by PCR. The RCN2 promoter primers were as follow: Primer 1: forward (5'-CAGTCCCAGCATGGCTC-3'), reverse (5'-GTCCACCAATGCGGTGC-3'); Primer 2: forward (5'-GCTGCACTTCCTGAGTTTG-3'), reverse (5'-TCCTACCGTCCATCAATGAG-3'); Primer 3: forward (5'-GAAACCTCCAGCC TCTCTC-3'), reverse (5'-AGCCATGCTGGGACTGC-3').

Tumorigenesis assay

Male BALB/c nude mice (4-5 weeks of age) were approved from the Central Laboratory of Animal Science, Wuhan University (Wuhan, China). HCCLM3 cells (5×10^6) stably transfected with RCN2-cDNA or empty vector were suspended in DMEM without FBS and were then injected into the armpits of 4 mice per group. The tumor volume was measured every 5 days after injection. The suspended cells $(3 \times 10^6, 100 \text{ ul})$ were injected into the tail veins of 4 mice per group. All the mice were sacrificed after 4 weeks, and the tumor and the lungs were removed. The tumors and lung metastasis were measured, and used for immunohistochemistry.

Immunoprecipitation and ubiquitination analysis

Huh7 and HCCLM3 cells were transfected MYC plasmid and RCN2 or vector. After 48 h, the cells were incubated with 10 μ M MG132 (HY-13259, MedChemExpress) for 8 h. MYC protein was immunoprecipitated by A/G beads (Life Technologies). And western blot analysis was used with Anti-ubiquitin antibodies or Anti-HA antibodies to detect MYC protein degradation.

Bioinformatics analysis

The HCC transcriptomic and clinical data were downloaded from The Cancer Genome Atlas (TCGA) liver cancer dataset from Genomic Data Commons Data Portal (https://portal.gdc.cancer.gov/) and Gene Expression Omnibus (GEO) database. And Genecard (https://www.genecards.org/) and ENCODE (http://genome.ucsc. edu/ENCODE/) programs were used to predict the transcription factors involved in the regulation of RCN2. R software (https://bioconductor. org/biocLite.R) were preferred to conduct single-gene gene set enrichment analysis (GSEA).

Statistical analysis

All statistical analyses were performed using SPSS 24.0 software. Quantitative data were presented as the mean \pm standard deviation of at least three independent experiments. Student's t-test for two groups and one-way ANOVA for multiple groups. The χ^2 test was used to analyze the relationship between RCN2 expression and the clinicopathological characteristics. Kaplan-Meier test from GEPIA (http://gepia.cancer-pku.cn/) were used to evaluate overall survivals, and the value of *P*<0.05 was considered statistically significant.

Result

Increased RCN2 level predicted poor prognosis in HCC patients

Quantitative real-time PCR was used to examine the mRNA levels of RCN2 in 40 paired HCC and adjacent non-tumor samples from Zhongnan Hospital of Wuhan University. 374 cases HCC samples and 50 cases normal samples from TCGA LICH datasets were downloaded to search RCN2 levels. We found that expression of RCN2 in tumor tissues were high-

er than those of matched non-tumor tissues (P<0.01, P<0.001, Figure 1A). The results were confirmed by WB and IHC (Figure 1B, 1C). Similarly, RCN2 expression was higher in diverse hepatoma cell lines than in the immortalized normal hepatocyte line LO2 (Figure 1D). Clinical data analysis revealed that a high level of RCN2 expression was significantly associated with tumor grade and cancer stage (P<0.05. P<0.01, P<0.001, Figure 1E; Table 1). In addition, analysis of transcriptome datasets of GSE40367 [19] suggested that RCN2 upregulated in HCC with metastasis (P<0.01, P< 0.001, Figure 1F). Moreover, Kaplan-Meier survival analysis of 364 HCC samples from TCGA revealed that patients with high levels of RCN2 had shorter overall survival. (P<0.001, Figure 1G).

RCN2 induced invasion and migration of HCC cells in vitro and in vivo

Previous study showed that down-regulated RCN2 suppress HCC cell proliferation [14]. In this study, we stably transfected with human RCN2-cDNA in two HCC cell lines, Huh7 and HCCLM3, which had different RCN2 expression. Both Huh7 and HCCLM3 cells were verified by WB. Compared with blank and negative controls, over-expressed RCN2 cells showed down-regulation of the epithelial markers E-cadherin, up-regulation of mesenchymal marker N-cadherin and Vimentin (Figure 2A). CCK8 assay confirmed that RCN2 was related to cell proliferation of Huh7 and HCCLM3 cells (P<0.01, Figure 2B). Transwell invasion assay and wound healing assay were used to evaluate the invasion and migration ability of various cell. The results in the low panel statistically revealed that over-expressed RCN2 in HCC cell increased cell invasion and migration (P<0.05, P<0.01, P<0.001, Figure 2C, 2D). Moreover, knockdown of RCN2 led to the opposing trends of HCC cells biological function (Additional file 1: Figure S1).

To further examine the effects of RCN2 in HCC tumorigenesis, HCCLM3 cells with up-regulated RCN2 were implanted subcutaneously and intravenously into Male BALB/c nude mice (n=4 per group). The volumes of xenograft tumor removed from mice injected by RCN2 overex-pression HCCLM3 cells were larger and more aggressive than negative control group



Figure 1. Increased expression of RCN2 in human HCC predicted poor prognosis. A. RCN2 mRNA levels from 40 paired HCC and pair-matched adjacent nontumor tissue and TCGA data. B. RCN2 protein levels in 6 paired human HCC specimens. C. Representative images of IHC staining with anti-RCN2 antibody in HCC and para-tumor tissues. D. RCN2 expression in HCC cell lines (Huh7, HCCLM3, SK-Hep1, HepG2, Hep3B) and the immortalized normal human hepatic cell (LO2). E. Differential levels of RCN2 across tumor grades and cancer stages from TCGA LICH datasets. F. RCN2 levels in metastatic and non-metastatic HCC samples from GSE40367. G. Kaplan-Meier analysis of the overall survival of 364 HCC patients from TCGA dataset. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NT, nontumor tissue; CC, colorectal cancer; HR, hazard ratio. **P*<0.05, ***P*<0.01, ****P*<0.001. Scale bar =50 µm.

Characteristics	Number of Cases	RCN2 expression		Pvalue
		High (n=20)	Low (n=20)	i value
Gender				0.723
Female	11	6	5	
Male	29	14	15	
Age				0.204
<65	18	7	11	
≥65	22	13	9	
Tumor Size (cm)				0.097
<5	26	10	16	
≥5	14	10	4	
HBV infection				0.527
No	20	9	11	
Yes	20	11	9	
AFP (µg/L)				0.327
<400	15	6	9	
≥400	25	14	11	
Cirrhosis				0.705
No	9	5	4	
Yes	31	15	16	
Tumor grade				0.197
I-II	16	6	10	
III-IV	24	14	10	
BCLC stage				0.025*
А	17	5	12	
B+C	23	15	8	
PVTT				0.658
No	34	16	18	
Yes	6	4	2	

 Table 1. Association of RCN2 expression with clinicopathologic parameters of HCC patients

AFP, alpha fetoprotein, BCLC, Barcelona Clinic Liver Cancer, PVTT, portal vein tumor thrombus. **P*<0.05.

(*P*<0.05, **Figure 2E**). IHC proved that RCN2, Ki67, E-cadherin, N-cadherin and Vimentin expressed in subcutaneous xenograft of mice were corresponding to cellular characteristics (**Figure 2F**). Meanwhile, more tumor cells metastasized to the lungs of nude mice were calculated microscopically in the RCN2 group (*P*<0.01, **Figure 2G**).

YY1 regulated the RCN2 expression at transcriptional level

To investigate whether transcription factor participated in the regulation of RCN2, we conducted a bioinformatics analysis via Genecard, ENCODE and GEO. The Venn plot predicted that the RCN2 promoter was combined to YY1 tran-

scription factor (Figure 3A). The person correlation analysis from TCGA LIHC indicated RCN2 was positively related to YY1 (R=0.67, P<0.001, Figure 3B). Additionally, YY1 was significantly upregulated in HCC (P<0.001, Figure 3C), and increased YY1 levels correlated with poor clinical outcomes in many cancers [20, 21]. Therefore, luciferase reporter vectors containing the wildtype (WT) and mutant (MUT) YY1 binding sequences of the RCN2 were constructed. The result showed the upregulated YY1 remarkably increased the luciferase activity of WT vector compared with MUT vector (P<0.001, Figure 3D). Furthermore, ChIP assays revealed that YY1 binded directly to the promoter region of RCN2 in Huh7 cells (P<0.001, Figure 3E). Otherwise, mRNA and protein levels of RCN2 were prominently decreased in knocking down YY1 HCC cells with YY1 siRNA (*P*<0.01, **Figure 3F**).

MYC was crucial for tumor progression modulated by RCN2 in human HCC cells

Gene set enrichment analysis (GSEA) on the 371 HCC samples from TCGA LIHC suggested that MYC was mediated by RCN2 (Figure 4A). Therefore, we demonstrated MYC protein level was regulated in RCN2^{+/-} Huh7 and HCCLM3 cells (Figure 2A, Additional file 1: Figure S1A), while knocking down MYC did not change RCN2 expression (Figure 4B). To further explore the roles of MYC in RCN2induced invasion and migration, MYC was knocked down in overexpressed RCN2 HCC cells with MYC siRNA. As a result, the levels of epithelial and mesenchymal markers were reversed contrasted of negative control groups (Figure 4C). Functionally, knocking down MYC decreased the proliferation and suppressed the invasive and metastatic abilities of HCC cells (P<0.05, P<0.01, P<0.001, Figure 4D-F).

RCN2 enhanced MYC protein stability via ubiquitin-proteasome pathway

To explore how RCN2 regulated MYC signaling, we first measured the MYC mRNA levels with qRT-PCR in RCN2 overexpression or knock-down Huh7 and HCCLM3 cells, compared with



Figure 2. Upregulated RCN2 expression induced invasion and migration of HCC cells in *vitro* and in *vivo*. A. Expression levels of RCN2, MYC, epithelial marker (E-cadherin), and mesenchymal markers (N-cadherin, vimentin) in Huh7 and HCCLM3 cells after transfection with RCN2 cDNA and empty vector. Cells without any treatment were used as the blank control. B. Proliferative ability for the HCC cells as indicated with CCK8. C. Invasion ability in various cells by Transwell invasion assay. Scale bar =100 μ m. D. Migration ability of indicated cells lines measured by Wound healing assay. Scale bar =100 μ m. E. Representative images and the volume of xenograft tumors. F. RCN2, Ki67 and EMT markers expression in xenograft tumor detected with IHC. Scale bar =50 μ m. G. Representative HE stained images of the lung metastasis model and the number of lung metastases after treatment as indicated. Scale bar =100 μ m. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NC, negative control; OD, optical density; Ki67, marker of proliferation Ki-67. **P*<0.05, ***P*<0.01.



Figure 3. RCN2 was regulated directly by the transcription factor YY1. A. Venn plot showing transcriptional factors in the promotor region of RCN2 from Genecard, ENCODE and GEO databases. B. Pearson correlation analysis of YY1 with RCN2 in TCGA LIHC dataset. C. YY1 expression in human HCC samples from TCGA LIHC dataset. D. Schematic illustration of YY1 binding site on the wild type and mutant RCN2 promotor. Fluorescence activity in Huh7 cells with

RCN2 wildtype and mutant promotor with YY1 pcDNA3.1. E. ChIP assay showing YY1 binding to RCN2 in Huh7 cells (Primer 1-3 were three different primers of RCN2 promoter). F. RCN2 expression levels in YY1-knockdown HCC cells. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IgG, immunoglobulin G; IP, immunoprecipitation with YY1; NC, negative control; siNC, scrambled small interfering RNAs; siYY1, small interfering RNAs targeting human YY1 gene. **P<0.01, ***P<0.001.



Figure 4. MYC was crucial for RCN2 induced invasion and migration of human HCC cells. A. Enrichment pathways with RCN2 overexpression group in GESA. B. Expression of RCN2, MYC in MYC-knockdown HCC cells. C. Expression of E-cadherin, N-cadherin, and vimentin in RCN2⁺ HCC cells after transfection with MYC siRNA and empty vector. D. Proliferative ability for the indicated cell lines with CCK8. E. Invasion ability in indicated cells evaluated by Transwell invasion assay. F. Migration ability of HCC cells measured by Wound healing assay. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NC, negative control; OD, optical density; siNC, scrambled small interfering RNAs; siMYC, small interfering RNAs targeting human MYC gene. **P*<0.05, ***P*<0.01, ****P*<0.001. Scale bar =100 µm.

the control cells. The results suggested RCN2 did not regulate MYC mRNA levels (Additional file 2: Figure S2A, S2B). Meanwhile, anti-RCN2 co-immunoprecipitation (co-IP) indicated that RCN2 did not directly interact with MYC protein (Additional file 2: Figure S2C). However, KEGG enrichment analysis in TCGA liver cancer dataset revealed that RCN2 may correlated to "Ubiguitin mediated proteolysis" (Additional file 2: Figure S2D). Besides, Pearson correlation analysis demonstrated that several deubiquitinating enzymes (USP28, USP36, USP7, USP22, USP37 and USP13), which have been reported to deubiquitinate and stabilize MYC [22-27], had high correlation coefficient with RCN2 (Additional file 2: Figure S2D). MYC was a short half-lived protein and tightly regulated through the ubiquitin-proteasome pathway [28]. We then employed protein synthesis inhibitor cycloheximide (CHX, 10 ug/ml) in control and overexpressed RCN2 HCC cells. The MYC protein half-life in overexpressed RCN2 cells were longer than in control cells (Figure 5A, 5B). Ubiquitination assays indicated that RCN2 overexpression decreased the levels of ubiquitinated MYC protein (Figure 5C). The proteasome inhibitor MG132 (10 µM) antagonized the reduction in MYC protein levels caused by knockdown of RCN2 (Figure 5D), confirming that RCN2 inhibits the degradation of MYC protein via ubiquitin proteasome pathway. These results all suggested that RCN2 enhanced the stability of MYC protein through suppressing ubiquitin proteasome system.

Discussion

The endoplasmic reticulum Ca²⁺ binding protein of 55 kDa (ERC-55) encoded by RCN2 was initially identified by Weis et al. in 1994 [29]. Previous research reported that knocking out RCN2 significantly suppressed tumor cell growth and proliferation via repression of EGFR-ERK pathway [14, 30]. However, the roles of RCN2 in invasion and migration remained unexplored.

In this study, we demonstrated that RCN2 was highly expressed in HCC compared with adjacent noncancerous tissue, especially in advanced tumor grade and cancer stage. Consistent with survival analysis, indicating that RCN2 relate to ability of invasion and migration in HCC cells. Our data showed that upregulated RCN2 provoked HCC cell invasion and migration and accelerated tumor growth and metastasis in *vivo*.

Furthermore, YY1 was proved as the positive transcription factor of RCN2. YY1 was a zincfinger protein belonging to the GLI-Kruppel familv that bonded to gene promoters and may be involved in the transcriptional of 7% of the total mammalian gene set [31, 32]. YY1 also named Ying Yang-1, stood for positivity and negativity in ancient Chinese philosophy, and was a reflection of the dual function in gene regulation [33]. YY1 facilitated tumor in a range of cancer types including lung, gastric, ovarian, bladder, renal and leukemia [34-39]. Previous studies had confirmed that YY1 played as a tumor stimulator in HCC progression [40, 41]. YY1 was also over-expressed in metastatic tumor cells and regarded as an inducer of cancer metastasis [42, 43]. YY1 interacted with protein arginine methyltransferase 7 (PRMT7) and resulted in the reduction of E-cadherin [44]. In prostate cancer cells, overexpression of YY1 could promote epithelial-mesenchymal transition by downregulating twist-related protein 1 through reducing heterogeneous nuclear ribonucleoprotein M (hnRNPM) expression [45]. Luciferase reporter and ChIP assays showed YY1 directly combined with the promoter region of RCN2. Moreover, we observed positive correlation between YY1 and RCN2 in TCGA LIHC database. And RCN2 was following reduced by knocking out the expression of YY1.

Interestingly, gene set enrichment analysis (GSEA) from TCGA LICH datasets showed RCN2 maybe induce to MYC signaling. MYC was a hallmark molecular feature of both the initiation and maintenance of tumorigenesis, and linked to epithelial-mesenchymal transition (EMT) and metastasis [46, 47]. We found that MYC expression was upregulated in RCN2⁺ HCC cells, while knocking down of MYC did not affect the RCN2 expression. Previous evidences declared that YY1 was a potent inhibitor of MYC transforming activity [48]. Nonetheless, MYC was activated by YY1 and modulating the activity of YY1 [49, 50]. Impressively, our research showed down-regulated MYC did not affect the RCN2 expression. Furthermore, knocking down MYC in RCN2⁺ HCC cells almost completely reversed RCN2 induced invasion and migration changes, suggesting that MYC was a downstream target of RCN2. Notably, negative consequences of co-IP between RCN2 and MYC



Figure 5. RCN2 enhanced MYC protein stability via ubiquitin-proteasome pathway. A, B. Western blot analysis (left) of MYC protein levels in indicated Huh7 and HCCLM3 cells treated with CHX (10 µg/ml) for 0 h, 1 h, 2 h and 3 h;

the curves (right) were measured by ImageJ according to grayscale value. C. HCC cells were transfected with RCN2 plasmids or vector plasmids, MYC plasmids and HA-Ub plasmids. After 48 h the cells were treated with MG132 (10 μ M) for 6 h. Anti-MYC antibody was used for IP. Anti-Ub and Anti-HA antibody was used for ubiquitination assays. D. Western blot analysis of MYC protein levels in indicated Huh7 and HCCLM3 cells treated with/without MG132 (10 μ M) for 6 h. CHX, cycloheximide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IP, immunoprecipitation; siNC, scrambled small interfering RNAs; siRCN2, small interfering RNAs targeting human RCN2 gene.

meant that RCN2 did not directly interact with MYC. Our results showed that RCN2 specifically stabilized MYC via inhibition of ubiquitin-proteasome system, and subsequently activated MYC signaling pathway in HCC cells. However, the clear mechanism of RCN2-mediated MYC stability needed to be further elucidated.

In conclusion, our study established that RCN2 played an oncogenic role in HCC, promoted the ability of cell proliferation, migration, and invasion in *vitro* and *vivo*. Besides, we validated YY1 participated in upstream mediation of RCN2, which accelerated tumor progression by stabilizing MYC. These findings suggested that RCN2 was a critical prognostic factor and capable to be a therapeutic target in HCC.

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The study protocol was in line with the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Hospital's Protection of. Human Subjects Committee. Informed consent to participate in the study was obtained from each patient that was recruited.

Disclosure of conflict of interest

None.

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Figure S1. Knockdown of RCN2 reduced invasion and migration of HCC cells. A. Expression levels of RCN2, MYC, epithelial marker (E-cadherin), and mesenchymal markers (N-cadherin, vimentin) in Huh7 and HCCLM3 cells in RCN-knockdown HCC cells and negative control group. B. Proliferative ability for the HCC cells as indicated with CCK8. C. Invasion ability in various cells by Transwell invasion assay. D. Migration ability of indicated cells lines measured by Wound healing assay. *P<0.05; **P<0.01; ***P<0.001.



Figure S2. A. The RCN2 mRNA levels in RCN2 overexpression or knockdown Huh7 and HCCLM3 cells. B. The MYC mRNA levels in RCN2 overexpression or knockdown Huh7 and HCCLM3 cells. C. Co-immunoprecipitation experiments were used to examine whether RCN2 interacted directly with MYC in HCC cells. D. GSEA demonstrated an enrichment of "Ubiquitin mediated proteolysis" in the RCN2 overexpression group. E. Pearson correlation analysis of RCN2 with several deubiquitinating enzymes (USP28, USP36, USP7, USP22, USP37 and USP13), which erased MYC ubiquitination. **P*<0.05; ***P*<0.01.

10

RCN2 expression

(log2(count+1))

12 14 (log2(count+1))

12

10

8

6

8

10 12 14

RCN2 expression

(log2(count+1))

10

8

6

4 2

6

14

p<0.001

8

p<0.001

6

8

10 12

RCN2 expression

(log2(count+1))