Research Article

Downregulation of CRABP2 Inhibit the Tumorigenesis of Hepatocellular Carcinoma In Vivo and In Vitro

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Cellular retinoic acid-binding protein 2 (CRABP2) binds retinoic acid (RA) in the cytoplasm and transports it into the nucleus, allowing for the regulation of specific downstream signal pathway. Abnormal expression of CRABP2 has been detected in the development of several tumors. However, the role of CRABP2 in hepatocellular carcinoma (HCC) has never been revealed. The current study aimed to investigate the role of CRABP2 in HCC and illuminate the potential molecular mechanisms. The expression of CRABP2 in HCC tissues and cell lines was detected by western blotting and immunohistochemistry assays. Our results demonstrated that the expression levels of CRABP2 in HCC tissues were elevated with the tumor stage development, and it was also elevated in HCC cell lines. To evaluate the function of CRABP2, shRNA-knockdown strategy was used in HCC cells. Cell proliferation, metastasis, and apoptosis were analyzed by CCK-8, EdU staining, transwell, and flow cytometry assays, respectively. Based on our results, knockdown of CRABP2 by shRNA resulted in the inhibition of tumor proliferation, migration, and invasion in vitro, followed by increased tumor apoptosis-related protein expression and decreased ERK/VEGF pathway-related proteins expression. CRABP2 silencing in HCC cells also resulted in the failure to develop tumors in vivo. These results provide important insights into the role of CRABP2 in the development and development of HCC. Based on our findings, CRABP2 may be used as a novel diagnostic biomarker, and regulation of CRABP2 in HCC may provide a potential molecular target for the therapy of HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most malignant cancers that occurred in liver [1]. Advanced therapeutic approaches have been administered in the prognoses of HCC; however, due to the unobvious pathognomonic symptoms, most HCC patients are initially diagnosed as advanced stage [2]. Surgical resection and liver transplantation are the therapeutic clinical treatment for HCC [3], while the 5-year recurrence rate of HCC is greater than 60%, and the survival rate and prognosis is poor [4–6]. Therefore, it is urgent to develop new therapies and identify novel therapeutic targets for HCC.

Cellular retinoic acid-binding proteins (CRABP2), belonging to intracellular lipid-binding proteins family, is a small cytosolic protein that contains 138 amino acid residues [7]. CRABP2 is a plasmonuclear shuttling protein, which transports retinoic acid (RA) to the nucleus and interacts with its receptor complex retinoic acid receptor (RAR) [8], acting as a coactivator of RAR. RAR by binding RA response element of target gene to regulate gene expression, CRABP2 is able to regulate cell proliferation, apoptosis, and metastasis by transporting retinoic acid to the nucleus [8–10]. It has been widely reported that abnormal CRABP2 expression change is associated with oncogenesis [11, 12]. The overexpression of CRABP2 has been reported in nonsmall cell lung cancer (NSCLC) [13], while CRABP2 is strongly associated with the occurrence of breast cancer. Feng et al. reported that CRABP2 can suppress invasion and metastasis of ER+ breast cancer by regulating the stability of Lats1 in vitro and in vivo [14]. However, few is known about the effects of CRABP2 in HCC.
Extracellular signal-regulated kinases (ERK) are members of mitogen-activated protein kinase (MAPK) super family [15, 16]. Activated via phosphorylation, ERK transduces extracellular signal into nucleus to trigger expression and transcription responses [17]. It has been widely accepted that ERK is tightly related with cell apoptosis and tumor growth [18]. Phosphorylated ERK (p-ERK) interact with downstream effectors-Bcl-2 family and caspase signaling pathway to adjust cell proliferation and apoptosis [19]. In fact, ERK is activated during the liver development [20]. Recent studies have found that ERK signaling pathway is the target for many regulators which are involved with HCC growth such as Castor zinc finger 1 [21] and IncIHS [22]. Additionally, by promoting the expression of vascular endothelial growth factor (VEGF), p-ERK enhance the angiogenesis of tumor tissues and accelerate the tumor growth subsequently [23, 24]. Vascular supply is closely related with tissue metabolic rate, while energy metabolism level directly affects the progression of tumor growth [25]. VEGF signaling pathway has been recognized as a key mediator in the process of HCC [26]. The mRNA expression levels of vascular endothelial growth factor A (VEGFA) in HCC was 6.95-fold higher when compared with HBsAg-negative healthy individuals [27].

In this study, we aim to elucidate the role of CRABP2 in HCC and the potential molecular mechanism involved in it. The expression of CRABP2 in HCC tissues was detected, while the effects of CRABP2 in HCC cell proliferation, apoptosis, and metastasis were assessed by CRABP2-shRNA transfection. In addition, we investigate the interaction between CRABP2 and VEGF signaling pathway in regulating HCC cell growth in vivo. Our study provides a new therapeutic target for HCC treatment.

2. Materials and Methods

2.1. Clinical Sample Collection and Cell Culture. From December 2015 to May 2018, HCC tissues and adjacent tissues were obtained from 58 patients who underwent HCC resection in The First Hospital of Jilin University and had not been administered radiotherapy and/or chemotherapy. Grade of HCC tissues was distinguished on the basis of AJC-C/UICC TNM staging system during pathological examination. All participants have signed an informed consent form in advance. The correlations between CRABP2 expression and HCC patient clinicopathological characteristics undergo HCC surgical removal.

| Clinical factor | CRABP2 expression |
|-----------------|-------------------|
|                 | Low expression (n = 22) | High expression (n = 36) | P value |
| Age (y)         |                  |
| <60             | 9                 | 15                | 0.955   |
| ≥60             | 13                | 21                |         |
| Gender          |                  |
| Male            | 14                | 21                | 0.689   |
| Female          | 8                 | 15                |         |
| Tumour size (cm)|                  |
| <5              | 10                | 9                 | 0.107   |
| ≥5              | 12                | 27                |         |
| AFP (ng/mL)     |                  |
| <20             | 9                 | 11                | 0.421   |
| ≥20             | 13                | 25                |         |
| TNM stage       |                  |
| I + II          | 13                | 8                 | 0.005** |
| III + IV        | 9                 | 28                |         |
| HBV infection   |                  |
| No              | 9                 | 10                | 0.863   |
| Yes             | 13                | 16                |         |
| Cirrhosis       |                  |
| No              | 15                | 12                | 0.01**  |
| Yes             | 7                 | 24                |         |
| Metastasis      |                  |
| No              | 10                | 7                 | 0.035*  |
| Yes             | 12                | 29                |         |

The low and high is graded by comparison of the mean Normal IHC scoring. *Statistically significant (P < 0.05), **Statistically significant (P < 0.01). *Statistically significant (P < 0.001).

2.2. Cell Transfection. Lentivirus vector-based shRNA technique was carried out to evaluate the function of CRABP2 knockdown in HCC cell. Two different human CRABP2 small hairpin RNA (shRNA) and a negative control lentivirus were designed and obtained from GeneChem (Shanghai, China). CRABP2-shRNA sequences are as follows: CRABP2-shRNA1: sense 5'-GGGAGAGUGAGAUUUGGUUU-3', antisense 5'-AUUUUAUUCACGUUCCTT-3'. CRABP2-shRNA2: sense 5'-GGGAGAGUGAUAUAUUAAATTTT-3', antisense 5'-UUUAUUUAUUCACGUUCCTT-3'.

2.3. CCK-8 Assay. CCK-8 assay was used to measure cell viability. Stable transfected cells were resuspended and seeded in a 96-well plate at 37°C (1000 cells/well). Cells were incubated for 4 h after adding 10 μL of CCK-8 solution (Sigma). Absorbance at 450 nm was measured with a spectrophotometer (SmartSpec 3000, Bio-Rad, CA, USA). The experiment was...
repeated at least 3 times and the cell viabilities were analyzed at 24 h, 48 h, and 72 h, respectively.

2.4. Flow Cytometry. Apoptosis of HepG2 after transfection was determined by flow cytometry with the Annexin V-FITC Apoptosis Detection kit. Stable transfected cells were washed with cold PBS for three times and made into single-cell suspension in 200 μL binding buffer containing 10 μL Annexin V-FITC and 5 μL PI-PE for 30 min in the dark. The cell apoptotic rate was analyzed by fluorescence-activated cell sorting (FACS) in a flow cytometer (LSR II, BD Biosciences, Franklin Lakes, NJ, USA).

2.5. EdU Staining. 5-ethyl-2′-deoxyuridine (EdU) staining was conducted to measure cell proliferation using Click-it™ EdU imaging kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Transfected cells were seeded in a 96-well plate (4000 cells/well) and then fixed with 4% paraformaldehyde for 15 min. After washed twice, cells were permeabilized with 0.5% Triton X-100 for 20 min and then incubated with Click-it™ cocktail reaction buffer for 30 min in dark. Subsequently, 5 μg/mL 4,6-diamidino-2-phenylindole(DAPI) was added for DNA staining. Cells were observed under a fluorescent microscope and analyzed by Image-Pro Plus image analysis software (Langham Creek, Houston, TX, USA).

2.6. Immunofluorescence. After fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 5% BSA, cells were incubated with primary antibodies: anti-CRABP2 (1 : 50, Abnova), anti-Ki-67 (1 : 1,000, Novoceastra). Anti-rabbit IgG Fab2 conjugated with Alexa Fluor 488 was used as a secondary antibody (1 : 200, Invitrogen). DAPI was added for DNA staining; the fluorescence was captured by a laser confocal microscope.

2.7. Transwell Assays. Migration and invasion of HepG2 cells was measured by transwell assays. For the cell invasion detection, stable transfected cells were transferred into serum-free medium for 12 h. The upper chambers of the transwell (MCEP24H48, Merck Millipore, USA) were coated with 50 μL Matrigel matrix (1 : 8, BD Biosciences) and incubated at 37°C until gel formed. Medium containing 10% fetal bovine serum was added into the lower chamber, and the serum-free DMEM into the upper chamber where cell suspension was added. After 48 h of incubation, the cells migrated or invaded to the membrane of the lower chamber were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Six nonoverlapping visual fields were randomly selected for cell counting under a light microscope. For the migration test, steps were the same, while no Matrigel matrix was used.

2.8. Tumor Xenograft Model. Four-week-old BALB/Nude SPF level nude mice were purchased from Vitalriver Inc. (China) and were bred under SPF-conditions in Jilin University. All animal experiment protocols were approved by the Institutional Animal Care and Use Committee of First Hospital of Jilin University (SXXK-JLU-2018-021) and performed in accordance with the approved protocol. Transfected HepG2 cells were subcutaneously injected into the left flanks of the nude mice. 200 mg/g of D-luciferin was injected into every mouse, and the bioluminescence was analyzed using a charge-coupled device camera (IVIS; Xenogen). Tumor volumes were measured every 5 days and calculated as follows: tumor volumes (mm³) = length × width² × 0.5. The mice were sacrificed by cervical dislocation at the 15th day, and tumors from each mouse were resected for immunofluorescence and immunohistochemistry staining.

2.9. Immunohistochemistry Staining. Tumor tissues from patients or mice were immersed in 10% formalin and embedded with paraffin, then cut into 4-μm-thick section. After deparaffinized in xylene and rehydrated in gradient concentration ethanol, sections were treated with 3% hydrogen peroxide and blocked with 5% BSA (Bull Serum Albumin). The slides were incubated with anti-Ki-67 (1 : 50, Abcam, Cambridge, UK) or anti-CRABP2 (1 : 50, Abcam, Cambridge, UK) antibody at 4°C overnight and homologous secondary antibody the next day. Sections were counterstained with hematoxylin and observed under an optical microscope.

2.10. Western Blotting. RIPA buffer (Bio-Rad, Hercules, CA, USA) was used to extract the whole protein from HCC cells. The protein concentrations were determined by BCA assay kit (Bio-Rad). Proteins (100 μg/lane) were electrophoresed by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto PVDF membranes (Bio-Rad). Membranes were blocked with 5% skimmed milk for one hour and then incubated with the following primary antibodies: anti-Bcl-2 (BD Biosciences), anti-Bax (BD Biosciences), anti-cleaved-caspase-3 (BD Biosciences), anti-CRABP2 (Cell Signaling Technology), anti-ERK (Abcam), anti-p-ERK (Abcam), anti-VGEF (Cell Signaling Technology), anti-VEGFR2 (Cell Signaling Technology), and anti-GAPDH (Cell Signaling Technology) at 4°C overnight. The next day, the membranes were incubated with Horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, Danvers, USA) for 2 h. ECL reagents were used to detect the immunoreactivity, and the protein bands were accessed by ImageJ software (National Institutes of Health, MD, USA). Relative expression levels of the indicated proteins were compared with GAPDH expression.

3. Results

3.1. Upregulated Expression of CRABP2 in HCC Tissues and HCC Cell Lines. To preliminarily investigate the relationship between CRABP2 expression and HCC, first, we employed immunohistochemistry and western blotting to detect the expression of CRABP2 in HCC tissues. As shown in Figure 1(a), the degree of CRABP2 staining showed an
increasing tendency with the malignancy of the HCC. The results of western blotting assays verified the same observation (Figure 1(b)). We further determined the expression of CRABP2 in HCC cell lines. As shown in Figure 1(c), western blotting results demonstrated that CRABP2 expression was also upregulated in HepG2, PLC/PRF5, and Huh7 cells when compared with human normal liver cells L-02. Accordingly, these results above indicated that the increased expression of CRABP2 may be associated with the malignancy of HCC.

3.2. ShRNAs Transfection Induced Downregulation of CRABP2 in HepG2 Cells. Considering the increased expression of CRABP2 in HCC tissues and cell lines, we transfected CRABP2-specific shRNAs into HepG2 cells to investigate the potential role of CRABP2 in HCC. After transfection, we employed immunofluorescence and western blotting assays to verify the interfering effects of the two CRABP2-shRNA we designed. As shown in Figure 2(a), the fluorescence intensity of CRABP2 decreased significantly after both CRABP2-shRNAs transfection. The protein expression of CRABP2 was downregulated when compared with the negative-control shRNA group (NC-shRNA) in both HepG2 and Huh7 cells (Figure 2(b)). These results suggest that CRABP2-shRNA effectively downregulated the CRABP2 expression in both HepG2 and Huh7 cells.

3.3. Knockdown of CRABP2 Inhibited Cell Proliferation, Migration, Invasion and Colony Formation of HCC Cell Lines. After HCC cell lines were transfected with CRABP2-shRNAs, we performed CCK8 assays and EDU staining to analyze the effect of CRABP2 silencing on cell proliferation. Downregulation of CRABP2 significantly suppressed cell
viability after 48 h and 72 h of transfection (Figures 3(a) and 3(b)), while EDU staining assays showed that CRABP2-shRNAs transfection decreased the EDU positive cells rate (Figures 3(c) and 3(d)). To study the effect of CRABP2 on HCC cell metastasis, we carried out transwell assays after CRABP2-shRNA transfection. As shown in Figures 3(e) and 3(f), cell migration and invasion abilities of HepG2 and Huh7 cells were dramatically suppressed by CRABP2 downregulation. Furthermore, as suggested in flow cytometry assays, CRABP2 suppression significantly enhanced the apoptotic rate of HepG2 and Huh7 cells (Figures 3(g) and 3(h)). Our data suggest that suppression of CRABP2 inhibited HCC cell proliferation and metastasis and play an inhibitory role in the regulation of apoptosis of HCC cells.

3.4. CRABP2 Downregulation Promote Apoptosis and Suppresses Survival, Migration, and Proliferation Related ERK-VEGF Pathway In Vitro. Based on the finding that CRABP2 downregulation inhibits cell proliferation partly due to the promotion of apoptosis, we suspected that CRABP2 downregulation may contribute to the expression of apoptotic-related genes. HepG2 and Huh7 cells transfected with CRABP2-shRNAs or NC-shRNA were used to test the protein expression of apoptosis and ERK-VEGF pathway (Figure 4(a)). Compared with the NC-shRNA groups, the protein levels of cleaved caspase-3 and Bax were clearly upregulated, while Bcl-2 was downregulated by CRABP2 downregulation. Furthermore, as suggested in flow cytometry assays, CRABP2 suppression significantly enhanced the apoptotic rate of HepG2 and Huh7 cells (Figures 3(g) and 3(h)). Our data suggest that suppression of CRABP2 inhibited HCC cell proliferation and metastasis and play an inhibitory role in the regulation of apoptosis of HCC cells.

Figure 2: CRABP2 expression decreased after CRABP2-shRNA transfection. (a) CRABP2 expression was analyzed by immunofluorescence staining in HepG2 cells transfected with shRNAs. (b) CRABP2 expression was quantified by immunofluorescence intensity. n = 8. (c) Western blotting was applied to determine the protein expression of CRABP2 in HepG2 cells after shRNA transfection. n = 8. One-way ANOVA was used in multigroup comparison. Scale bar = 20 μm. All data are presented as mean ± SEM. **P < 0.01 vs. NC-shRNA.
Figure 3: Continued.
3.5. Downregulation of CRABP2 Inhibits Tumorigenesis and Angiogenesis In Vivo. To further confirm the biological role of CRABP2 on tumor growth in vivo, we established a xenograft model by injecting CRABP2-shRNAs transfected HepG2 cells into nude mice subcutaneously. Tumor volumes were measured every 5 days, and tumors were exercised and weighted after 15 days of injection. The results demonstrated that the downregulation of CRABP2 dramatically suppressed tumor growth and angiogenesis in vivo.
Figure 4: Continued.
tumor growth in vivo compared with NC-shRNA group (Figures 5(a) and 5(b)). With the CRABP2 expression decreased in tumor tissue, Ki-67, the proliferation marker, decreased as well. Furthermore, immunohistochemical staining results of CD31 showed that CRABP2 knockdown leads to the decreased vessel density within tumors xenografts (Figure 5(c)). These results suggest that CRABP2 downregulation inhibits HCC tumorigenesis in vivo.

4. Discussion

HCC is the fifth most common tumor occurring in the liver and is one of the leading causes of cancer-related deaths [28]. However, the pathogenesis of HCC is still not completely understood. Despite the effectiveness of advanced surgery with radio-/chemotherapy, about 80% of HCC patients are diagnosed with advanced stage preliminarily and unable to accept surgical resection [29]. Besides, the rate of postoperative recurrence and metastasis probability of HCC is high [30]. Thus, new strategies for the diagnosis and treatment of this malignant disease are urgently needed. CRABP2, a retinoic acid signaling component, has either oncogenic or tumor-suppressive effects that may depend upon the type of malignancies. However, expression status and the role of CRABP2 in HCC are still unclear. By immunohistochemical and western blot analysis, we found that the level of CRABP2 expression was higher in HCC tissues than in the corresponding peri-HCC tissues. Results of TMA analysis confirmed that CRABP2 expression was positively correlated with pathological grade in patients with HCC. Based on experiments of the knockdown strategy with lentivirus system, we found that the downregulation of CRABP2 could inhibit proliferation of HCC cell in vitro and in vivo. These data imply that CRABP2 may act as an oncogene in HCC.

CRABP2 contains a retinoic acid-binding domain and sensitizes the cellular response to the antiproliferative signaling by retinoic acid. CRABP2 is reported to be related with the development of neuroblastoma, non-small cell lung cancer (NSCLC), ovarian cancers, breast cancer, and so on [13, 31]. Yu et al. reported that CRABP2 enhances pancreatic cancer cell migration and invasion by stabilizing interleukin 8 expression [32], and Liu et al. reported CRABP2 altering retinoic acid signaling is associated with poor prognosis in glioblastoma [33]. CRABP1, another member of the RABP-binding protein family, was reported to be upregulated in HCC-prone HBx Tg mice markedly [34]. However, it is not clear whether CARBP2 affects the HCC. In the present study, we observed that CRABP2 upregulated in HCC tissues and cell lines significantly. To explore the biological function of CRABP2 in HCC progression, we suppressed CRABP2 expression by lentivirus vector-based shRNA transfection. The two different CRABP2-shRNA we designed were able to knockdown CRABP2 expression steadily in HepG2 cells. Our data showed that CRABP2 suppression led to significant inhibition of cell proliferation, invasion, and migration. Furthermore, CRABP2 downregulation increased HepG2 cell apoptosis. In addition, CRABP2 knockdown suppressed tumor growth in nude mice xenografts as well. These data indicate that suppression of CRABP2 inhibited the progression of HCC, and CRABP2 may play a tumor promoter role in human HCC progression. To our knowledge, our study is the first to report the expression change of CRABP2 in HCC tissues and cell lines. Therefore, the mechanism by which CRABP2 regulated HCC cell growth and metastasis requires further investigation.

It has been reported that ERK is overexpressed and hyperactive in various types of cancer. Almost half of known human tumor cell lines and a large number of human carcinoma in situ are related with ERK activation [35]. Metastasis and apoptosis of tumors requires specific intracellular signaling cascade activations, while ERK signaling pathway is a crucial part [19]. Previous reports show that the phosphorylation levels of ERK were significantly upregulated in laryngeal cancer and contributed to tumor progression [36]. Numerous of reports suggest that ERK pathway...
participates in the progression of HCC [37]. p-ERK levels were increased in medium cultured HCC cells which were isolated from HCC patients [38]. Chen et al. reported baikain inhibits HCC invasion and migration via the suppression of the ERK pathway [39]. Our western blotting results showed that CRABP2 knockdown significantly decreased p-ERK/ERK ratio in HepG2 cells. Bcl2 and caspases family are the downstream pathway through which ERK regulates apoptosis. We found that cleaved caspase-3 and Bax increased, while Bcl2 decreased after CRABP2-shRNAN transfection. However, the interaction mechanism of ERK pathway and CRABP2 in HCC was remained unclear which require further exploration.

Our in vivo data revealed that CRABP2 suppression also inhibits angiogenesis in tumors xenograft. Angiogenesis is essential for tumor development and progression, tumor growth and survival depend on the oxygen and nutrients provided by blood vessels [40]. VEGF and VEGFR are important mediators for tumor angiogenesis in various cancers [41]. VEGF acts with VEGFR in vascular endothelial cells to promote differentiation and proliferation of vascular endothelial cells, resulting in angiogenesis, which is closely related to the growth, metastasis, and prognosis of cancers [27]. Studies have shown that ERK regulates angiogenesis by interacting with VEGF pathway [42]. So we hypothesized that CRABP2 may inhibit the angiogenesis of HCC through

**Figure 5:** Effects of CRABP2 on tumor growth in vivo. HpeG2 cells transfected with two different CRABP2-shRNA were injected into nude mice (n = 13 for each group). Representative images of tumors dissected from mice are shown in (a), and tumor volume (b) was measured every other 5 days. Representative images of CRABP2, Ki-67, CD31 staining after immunohistochemistry are shown in (c). Scale bar = 100 μm. One-way ANOVA was used in multigroup comparison. All data are presented as mean ± SEM. ***P < 0.001 vs. NC-shRNA.
ERK/VEGF pathway. We speculate that the downregulation of CRABP2 was involved in ERK/VEGF induced angiogenesis in HCC.

Taken together, CRABP2 was upregulated in HCC, which suggested a role for CRABP2 in the promotion of HCC growth and CRABP2 could serve as a new prognostic predictor for HCC, as well as a potential therapeutic target.

Data Availability

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

Ethical Approval

This study was approved by the Ethics Committee of the First Hospital of Jilin University (20150801A). Informed consent was obtained from all individual participants included in the study.

Consent

All authors agreed for the publication of this paper.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors’ Contributions

Qingmin Chen conceived the experiments; Ludong Tan contributed to research data; Qingmin Chen drafted the manuscript; Zhe Jin contributed to technical assistance; Yahui Liu and Ze Zhang revised the manuscript and supervised the analysis.

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