CRP-1 promotes the malignant behavior of hepatocellular carcinoma cells via activating epithelial-mesenchymal transition and Wnt/β-catenin signaling

SHIXIONG LEI[1*], XILIN DU[2], KAI TAN[2*], XIAOJUN HE[2*], YEJING ZHU[2], SHOUJIE ZHAO[2], ZHENYU YANG[2] and GANG DOU[2]

Departments of [1]Interventional Medicine and [2]General Surgery, The Second Affiliated Hospital of Air Force Military Medical University, Xi'an, Shaanxi 710038, P.R. China

Received December 28, 2022; Accepted April 18, 2023

DOI: 10.3892/etm.2023.12013

Abstract. Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. It has been reported that cysteine rich protein 1 (CRP-1) is dysregulated in several types of human cancer; however, its role in HCC is poorly understood. Therefore, the current study aimed to investigate the role of CRP-1 in HCC. Western blotting and reverse transcription-quantitative PCR results showed that CRP-1 was upregulated in HCC cell lines. Furthermore, for *in vitro* experiments, CRP-1 was knocked down and overexpressed in the HCC cell lines Hep 3B2.1-7 and BEL-7405, respectively. c-Myc and proliferating cell nuclear antigen upregulation, and cleaved caspase 3 and poly(ADP-ribose) polymerase downregulation suggested that CRP-1 silencing could inhibit the proliferation and colony-forming ability of HCC cells, and induce apoptosis. In addition, CRP-1 overexpression promoted the malignant behavior of HCC cells and induced epithelial-mesenchymal transition (EMT), as verified by E-cadherin downregulation, and N-cadherin and vimentin upregulation. Additionally, CRP-1 overexpression promoted the nuclear translocation of β-catenin, and activated the expression of cyclin D1 and matrix metalloproteinase-7. Furthermore, inhibition of Wnt/β-catenin signaling, following cell treatment with XAV-939, an inhibitor of the Wnt/β-catenin signaling pathway, abrogated the effects of CRP-1 on enhancing the proliferation and migration of HCC cells. These findings indicated that the regulatory effect of CRP-1 on HCC cells could be mediated by the Wnt/β-catenin signaling pathway. Overall, CRP-1 could promote the proliferation and migration of HCC cell lines, partially via promoting EMT and activating the Wnt/β-catenin signaling pathway.

Correspondence to: Professor Xilin Du, Department of General Surgery, The Second Affiliated Hospital of Air Force Military Medical University, 1 Xinsi Road, Baqiao, Xi'an, Shaanxi 710038, P.R. China
E-mail: duxl0705@fmmu.edu.cn

[*]Contributed equally

Key words: hepatocellular carcinoma, cysteine rich protein 1, proliferation, migration, epithelial-mesenchymal transition, Wnt/β-catenin signaling pathway

Introduction

Primary liver cancer is one of the most common malignant tumors in clinical practice, with the fifth and third highest incidence and mortality rate, respectively, among malignant tumors worldwide (1,2). Therefore, it is considered extremely harmful. In addition, primary liver cancer includes several pathological types, among which hepatocellular carcinoma (HCC) is the most predominant type, accounting for ~80-90% of all liver cancer cases (3), closely followed by cholangiocarcinoma and mixed hepatocellular cholangiocarcinoma (4). In China, HCC is the fourth most common type of tumor (5), accounting for 75-85% of all liver cancer cases annually (6). The difficulty of early diagnosis of HCC results in the diagnosis of patients commonly in the middle to late stages of the disease, thus hindering the timely treatment of HCC in clinical practice (7). Currently, surgery and chemotherapy are the most common treatment approaches for HCC. However, due to the complex etiology of HCC and its high metastasis rate, surgical resection commonly leads to poor results and poor prognosis (8). Another difficulty is the resistance of HCC to anti-cancer drugs (9). Therefore, investigating the molecular mechanisms underlying the development of HCC and identifying potential biomarkers and targets for the development of novel treatment strategies for HCC are of great importance.

Cysteine rich protein 1 (CRP-1), together with cysteine- and glycine-rich protein-1, rhombotin-1, rhombotin-2 and rhombotin-3, belong to the LIM/double zinc finger domain family (10,11). CRP-1 was initially identified as an intracellular zinc transporter and absorption protein (12). Further studies showed that CRP-1 was involved in the host's immune response (13). Previous studies also revealed that CRP-1 was aberrantly expressed in several types of cancer, including osteosarcoma (14), breast cancer (15), cervical cancer (16), thyroid carcinoma (17), prostate cancer (18), pancreatic

cancer (19) and colorectal cancer (20). However, the role of CRP-1 in cancer remains controversial. He *et al* (21) demonstrated that CRP-1 was upregulated in human colorectal cancer, while CRP-1 knockdown inhibited the invasion and migration ability of colon cancer cells. The above findings suggested that CRP-1 could be a novel biomarker indicating poor prognosis in colorectal cancer. The inhibitory role of CRP-1 knockdown was also found in cervical and thyroid cancer (16,17). By contrast, CRP-1 was considered as an oncogene in breast cancer and its expression restrained the malignant potential of breast cancer cells (15). The above studies suggested that CRP-1 exerted a diverse role in different types of cancer. However, the effect of CRP-1 on HCC has not been previously investigated.

The present study aimed to investigate the expression profile, function and possible underlying mechanism of CRP-1 in HCC. Therefore, bioinformatics analysis, using the Gene Expression Profiling Interactive Analysis (GEPIA) and UALACAN databases, and Kaplan-Meier survival analysis, were performed to detect the expression of CRP-1 in HCC and evaluate its effect on the survival of patients with HCC, respectively. Further *in vitro* experiments were also carried out to explore the role of CRP-1 in the proliferation, migration, invasion and epithelial-mesenchymal transition (EMT) of HCC cells, as well as to uncover its possible underlying mechanism of action.

Materials and methods

Differential gene expression (DEG) and survival analysis. DEG analysis in liver hepatocellular carcinoma (LIHC) was performed using the GEPIA online database (http://gepia.cancer-pku.cn/detail.php). DEG was considered significant when $\log_2$ fold of change (FC)>1 or $\log_2$FC<-1 ($P<0.05$). Volcano plots of DEGs and their chromosomal locations were then illustrated. Survival analysis was conducted using Kaplan-Meier survival analysis (http://kmplot.com/analysis/) with log-rank test.

Cell lines and cell culture conditions. The human hepatoma cell lines Huh-7, MHCC97, Hep3B2.1-7, PLC/PRF/5 and BEL-7405 were purchased from iCell Bioscience, Inc. MHCC97 and Huh-7 cells were cultured in a standard Dulbecco modified Eagle medium (DMEM) (Wuhan Service bio Technology Co., Ltd.), while Hep3B2.1-7 and PLC/PRF/5 cells in standard minimum essential medium (MEM; Beijing Solarbio Science & Technology Co., Ltd.). Finally, BEL-7405 cells were cultured in standard RPMI-1640 medium (Beijing Solarbio Science & Technology Co., Ltd.). All cells were grown at 37˚C in a humidified incubator with 5% CO_2.

Plasmid transfection. Based on the expression levels of CRP-1 in HCC cell lines, BEL-7405 and Hep 3B2.1-7 cells were selected to establish stable CRP-1-overexpressing and silencing cells, respectively. The CRP-1 overexpression plasmid and the corresponding control vector or short hairpin RNA (shRNA) targeting CRP-1 and non-targeting shRNA (shNC) were transfected into BEL-7405 or Hep3B2.1-7 cells, respectively, using Lipofectamine® 3000. Stably CRP-1-overexpressing or depleted cells were selected following treatment with 400 μg/ml G418.

Inhibition of the Wnt/β-catenin signal pathway. For the inhibition experiments, the stable CRP-1-overexpressing cell lines were treated with 10 μM XAV-939 (Shanghai YuanYe Biotechnology Co., Ltd.) for 24 h for further analysis.

Cell Counting Kit-8 (CCK-8) assay. Cell viability was assessed using a CCK-8 kit (Nanjing KeyGen Biotech Co., Ltd.). Briefly, the stably transfected BEL-7405 and Hep3B2.1-7 cells were inoculated into 96-well plates at a density of $1x10^4$ cells/well. Cell viability was then determined at 0, 24, 48 and 72 h following seeding, according to the manufacturer's instructions.

Assessment of cell apoptosis. Cell apoptosis was assessed using an Annexin V-FITC/PI Apoptosis Detection Kit (Nanjing KeyGen Biotech Co., Ltd.). Briefly, the stably transfected Hep3B2.1-7 cells were resuspended in 500 μl binding buffer and were then supplemented with 5 μl Annexin V-FITC and 5 μl PI. Subsequently, the samples were gently vortexed to mix reagents and incubated for 5-15 min at room temperature in the dark. Emitted fluorescence was quantified using the NovoCyte flow cytometer (ACEA Bioscience, Inc.).

Transwell assay. To evaluate cell migration, HCC cells at a density of $1.5x10^4$ cells/well were cultured in the upper chamber of a Transwell insert (Anhui Labselect Technology Co., Ltd.). The lower chamber was filled with 800 μl medium supplemented with 10% FBS. Following incubation for 24 h, cells in the upper chamber were removed followed by fixing with 4% paraformaldehyde (PFA; Shanghai Aladdin regents Co. Ltd.) and staining with 0.1% crystal violet solution (Amresco, LLC). The migrated cells were counted and their images were captured under a light microscope (Olympus Corporation).

Wound healing assay. Cell migration was assessed using wound healing assays. The stably transfected BEL-7405 and Hep3B2.1-7 cells were pre-treated with serum-free medium supplemented with 1 μg/ml mitomycin C (MilliporeSigma) for 1 h. When cells reached 90% confluence, a wound was then created via scratching the cell monolayer with a 200-μl pipette tip. The average migration distance was measured at 24 h.

Colony formation assay. Stably transfected BEL-7405 and Hep3B2.1-7 cells were inoculated in 35-mm culture dishes at a density of 200 cells/dish for two weeks. The cells were then fixed with 4% PFA (MilliporeSigma) and the formed colonies were stained with Wright-Giemsa staining (Nanjing KeyGen Biotech Co., Ltd.).

Immunofluorescence staining. Immunofluorescence staining was performed on PFA-fixed cell climbing sheets. Briefly, cells were permeabilized with 0.1% tritonX-100 (Beyotime Institute of Biotechnology) for 30 min. Following washing three times in PBS for 5 min each, cells were blocked with 1% BSA for 15 min, incubated with primary antibody against β-catenin (ABclonal Biotech Co., Ltd.) at 4˚C overnight, followed by incubation with secondary Cy3-conjugated anti-rabbit IgG (Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h at room temperature.

Cell nuclei were stained with 4',6-diamidino-2-phenylindole (Shanghai Aladdin regents Co. Ltd.).

Western blot analysis. The whole cell lysates and nuclear proteins were extracted from cells using a Western/IP lysis buffer and a nuclear protein extraction kit (both from Beyotime Institute of Biotechnology), respectively. The proteins were separated by SDS-PAGE (Beyotime Institute of Biotechnology) and were then blotted onto PVDF membranes (MilliporeSigma). Subsequently, membranes were blocked for 1 h in 5% non-fat milk, followed by incubation with primary antibodies overnight at 4°C and then with the corresponding secondary antibodies for 45 min at 37°C. The bands were visualized using an enhanced chemiluminescence kit (Beyotime Institute of Biotechnology). The primary antibodies used were as follows: Anti-CRP-1 (dilution, 1:500; cat. no. A7548), anti-β-catenin (dilution, 1:1,000; cat. no. A19657; both from ABclonal Biotech Co., Ltd.), anti-c-Myc (dilution, 1:500; cat. no. BA1284-2), anti- proliferating cell nuclear antigen (PCNA; dilution, 1:1,000; cat. no. BM0104), anti-cleaved caspase 3 (dilution, 1:1,000; cat. no. M00334-7), anti-E-cadherin (dilution, 1:3,000; cat. no. BM3903), anti-N-cadherin (dilution, 1:500; cat. no. BA0673), anti-vimentin (dilution, 1:1,000; cat. no. PB9359), anti-cyclinD1 (dilution, 1:1,000; cat. no. PB0403), anti-matrix metalloproteinase 7 (MMP-7; dilution, 1:1,000; cat. no. PB9037), anti-histone H3 (dilution, 1:1,000; cat. no. A12477-2), anti-β-actin (dilution, 1:1,000; cat. no. BA2305; all from Wuhan Boster Biological Technology, Ltd.) and anti-cleaved poly(ADP-ribose) polymerase (PARP; dilution, 1:1,000; cat. no. #5625; Cell Signaling Technology, Inc.). The secondary antibodies used were the following: Goat anti rabbit-IgG (dilution, 1:5,000; cat. no. A0208) and goat anti-mouse IgG (dilution, 1:5,000; cat. no. A0216; both from Beyotime Institute of Biotechnology).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cells using the TRIpure kit (Bioteke, Beijing, China) and its concentration was quantified by NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific, Inc.). cDNA was synthesized using the BeyoRT II M-MLV reverse transcriptase kit (Beyotime Institute of Biotechnology). For qPCR analysis, the SYBR Green PCR Master Mix kit (Beijing Solarbio Science & Technology Co., Ltd.) was utilized. The relative gene expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (22). The primer sequences for CRP-1 were as follows: forward, 5'-AAGTGTCCCAAG TGCAACAA-3', and reverse, 5'-CGTCTTCCCACATTTCTC G-3'; β-actin: forward, 5'-GGCACCCAGCACAATGAA-3', and reverse, 5'-TAGAAGCATTTGCGGTGG-3'.

Statistical analysis. All statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software, Inc.). The differences between two groups were compared using unpaired Student's t-test. The differences among more than 2 groups were compared using one-way ANOVA, followed by Tukey's multiple comparison. For cell viability results, a two-way ANOVA with Tukey or Sidak's post-hoc test was run. Each experiment was replicated at least 3 times. Data are expressed as the mean ± standard deviation (SD). $P<0.05$ was considered to indicate a statistically significant difference.

Results

CRP-1 expression in human HCC. First, DEGs were analyzed in the GEPIA database. Therefore, a total of 2,224 DEGs, including 730 upregulated and 1,478 downregulated ones, were illustrated in volcano plots (Fig. 1A). The chromosomal location of DEGs is shown in Fig. 1D. Currently, the members of the LIM/double zinc finger domain family have gained tremendous attention due to their enriched cysteine LIM structure domain, which has been proved to be involved in tumor formation (23). Herein, the members of the LIM protein family were screened in the Pfam database (http://pfam.xfam.org/). The median expression levels of the LIM family members in tumor and normal tissues in the LIHC dataset are presented in Fig. 1B, while their log-fold-changes in Fig. 1C. Among them, CRP-1 exhibited the most obvious differential expression in the GEPIA database between tumor (n=369) and normal tissues (n=169; Fig. 1D).

CRP-1 is highly expressed in HCC cell lines. Subsequently, the expression levels of CRP-1 were also compared between tumor and normal liver tissues in the UALACAN database. The results revealed that CRP-1 was remarkably upregulated in HCC tissues compared with adjacent normal tissues (Fig. 2A). Kaplan-Meier survival analysis showed that patients with high CRP-1 expression levels had worse overall survival compared with those with low levels (Fig. 2B). In addition, western blot analysis and RT-qPCR analysis demonstrated that CRP-1 was differentially expressed in five HCC cell lines, namely BEL-7405, PLC/PRF/5, Huh-7, Hep3B2.1-7 and MHCC97. Therefore, Hep3B2.1-7 cells expressed the highest CRP-1 levels, BEL-7405 cells the lowest, and PLC/PRF/5, Huh-7 and MHCC97 cells moderate ones (Fig. 2C). Subsequently, Hep3B2.1-7 and BEL-7405 cells were transfected with specific shRNAs targeting CRP-1 (sh-Control; sh-CRP-1#1/2) and CRP-1 overexpression plasmids, respectively. The transfection efficiency was verified by RT-qPCR and western blot analysis (Fig. 2D and E).

CRP-1 regulates the proliferation and apoptosis of hepatoma cells. CRP-1-overexpressing BEL-7405 cells and CRP-1-depleted Hep3B2.1-7 cells were employed to investigate the biological function of CRP-1 in HCC *in vitro*. Therefore, CRP-1 silencing reduced the viability of Hep3B2.1-7 cells, as verified by CCK-8 assays. The opposite trend was observed in CRP-1-overexpressing BEL-7405 cells (Fig. 3A). To gain further insights into the mechanism underlying the effect of CRP-1 on HCC cell proliferation, the protein expression levels of PCNA and c-Myc were detected using western blot analysis. The results showed that the expression levels of both PCNA and c-Myc were notably decreased in CRP-1-depleted HCC cells. By contrast, CRP-1 overexpression yielded the opposite results (Fig. 3B). Furthermore, colony formation assays demonstrated that the number of colonies was significantly reduced in CRP-1-silenced Hep3B2.1-7 cells compared with the control group. However, the number of formed colonies was markedly higher in CRP-1-overexpressing BEL-7405 cells compared with control cells (Fig. 3C and D). In addition, the apoptosis rate of CRP-1-silenced Hep3B2.1-7 cells was significantly higher compared with that of control cells (Fig. 3E). The above finding was also supported by the protein expression

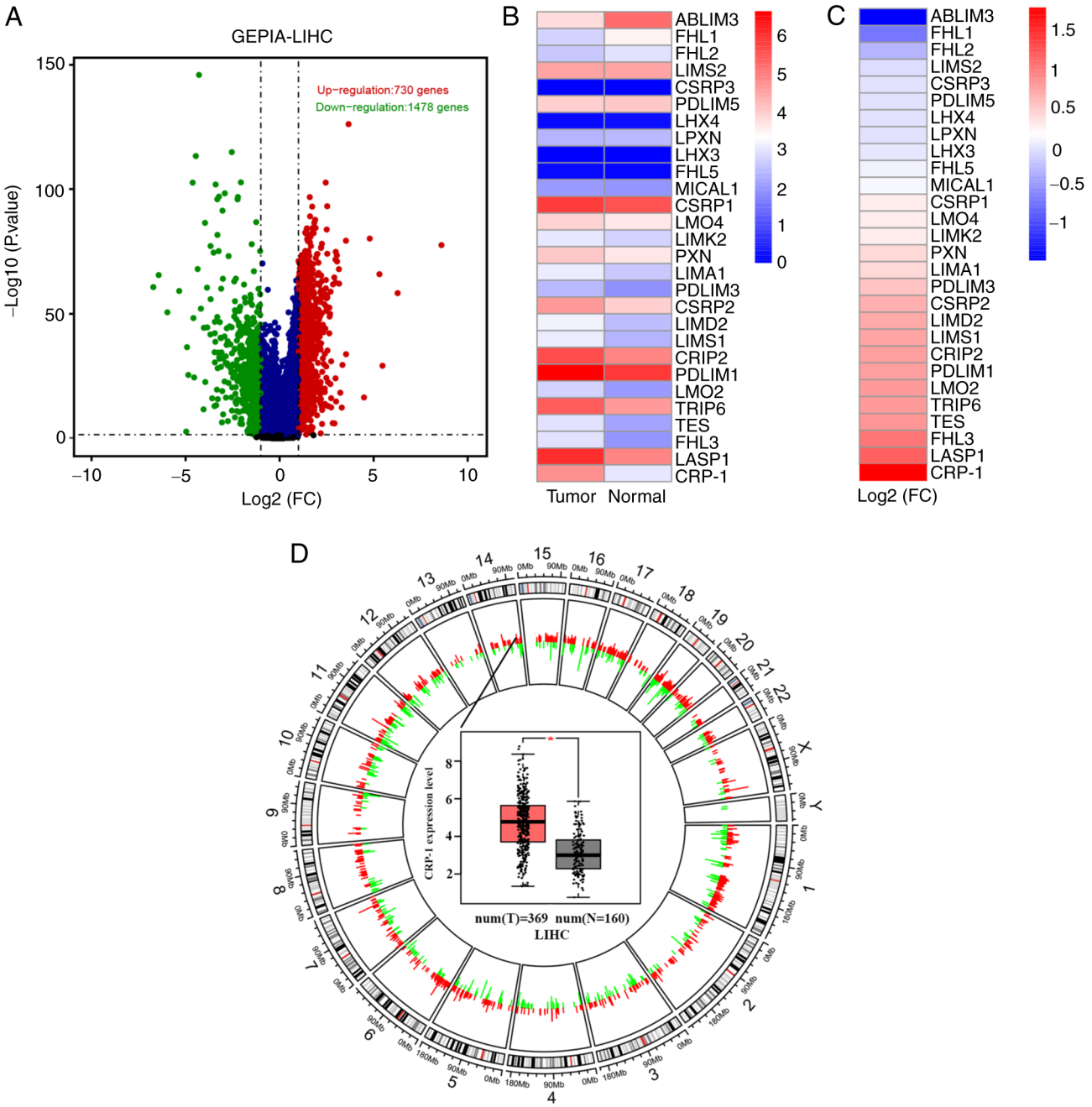

Figure 1. CRP-1 expression in human hepatocellular carcinoma. (A) Volcano plots of DEGs in the LIHC dataset downregulated from the Gene Expression Profiling Interactive Analysis database are shown. (B) The median expression levels of LIM/double zinc domain family members in normal and LIHC tissues. (C) The expression values [$\log_2$(fold-change)] of LIM protein family members in the GEPIA-LIHC dataset are shown. (D) The chromosomal distribution of DEGs is presented. The black line indicates the chromosomal distribution of CRP-1, while the central circle the difference in the expression levels of CRP-1 between normal and LIHC tumor tissues, according to the GEPIA online platform. CRP-1, cysteine rich protein 1; LIHC, liver hepatocellular carcinoma; DEGs, diffrentially expressed genes; GEPIA, Gene Expression Profiling Interactive Analysis.

levels of cleaved caspase 3 and cleaved PARP (Fig. 3F). The aforementioned results suggested that CRP-1 upregulation could promote the proliferation and survival of HCC cells.

CRP-1 regulates the migration, invasion and EMT of hepatoma cells. The wound healing assay results showed that CRP-1 knockdown attenuated wound healing compared with control cells, while CRP-1 overexpression enhanced the migration ability of BEL-7405 cells (Fig. 4A). In line with the above results, Transwell assays confirmed that CRP-1 silencing suppressed the invasion of Hep3B2.1-7 cells (Fig. 4B). By contrast, CRP-1-overexpressing cells exhibited a more aggressive invasion potential compared with control cells (Fig. 4B). Subsequently, the association between CRP-1 and EMT in HCC cells was investigated. The results demonstrated that CRP-1 overexpression promoted EMT process in BEL-7405 cells, accompanied by E-cadherin downregulation, and N-cadherin and vimentin upregulation. However, CRP-1 knockdown reversed these effects (Fig. 4C).

Effect of CRP-1 on the Wnt/β-catenin signal pathway. It has been reported that the aberrant activation of the Wnt/β-catenin

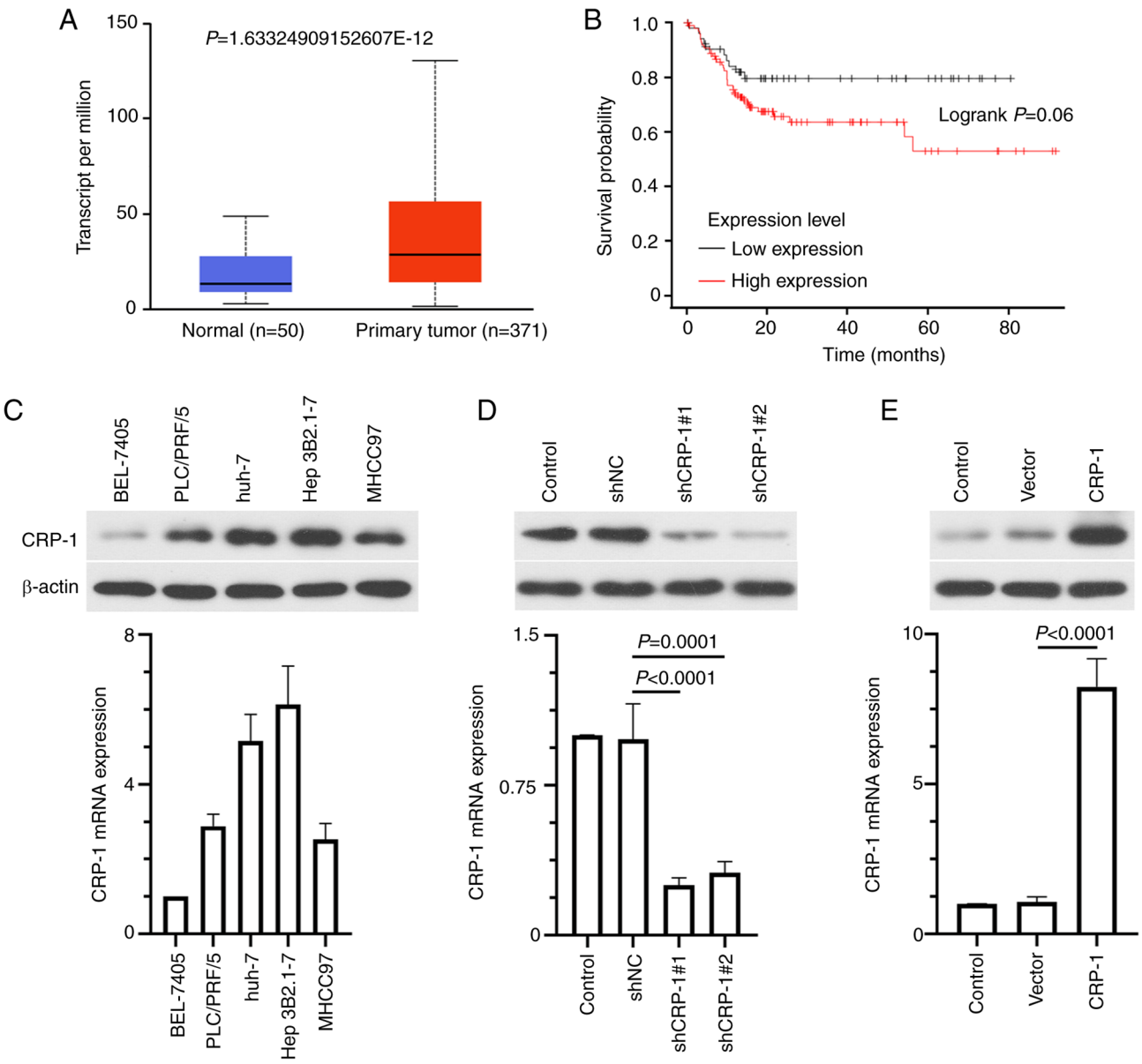

Figure 2. CRP-1 is significantly upregulated in hepatocellular carcinoma cell lines. (A) Data from the UALCAN database showed that CRP-1 was upregulated in liver cancer tissues compared with normal ones. (B) The Kaplan-Meier survival analysis of CRP-1 expression is presented. (C) The mRNA and protein expression levels of CRP-1 in hepatoma cell lines were assessed by RT-qPCR and western blot analysis, respectively. Stably (D) CRP-1-overexpressing or (E) -silencing cell lines were established in BEL-7405 or Hep 3B2.1-7 cells, respectively. The mRNA and protein expression levels of CRP-1 in BEL-7405 (CRP-1 overexpression) and Hep 3B2.1-7 (CRP-1 silencing) cells were detected by RT-qPCR and western blot analysis, respectively. Data are expressed as the mean ± SD of three independent experiments. CRP-1, cysteine rich protein 1; RT-qPCR, reverse transcription-quantitative PCR.

pathway plays a key role in the development of HCC (24). Therefore, the present study aimed to investigate whether CRP-1 was involved in the regulation of Wnt/β-catenin signaling in HCC cells. The effect of CRP-1 overexpression or silencing on enhancing or inhibiting the nuclear localization of β-catenin in HCC cells was verified by immunofluorescence staining (Fig. 5A). Therefore, CRP-1 overexpression significantly enhanced the nuclear expression β-catenin and that of the Wnt/β-catenin signaling-related downstream target-genes, cyclin D1 and MMP-7 (Fig. 5B). However, the expression levels of the above proteins were reduced in CRP-1-depleted HCC cells (Fig. 5B). To determine whether the Wnt/β-catenin pathway was involved in the CRP-1 induced HCC cell proliferation and migration, cells were treated with XAV-939, a Wnt/β-catenin signaling inhibitor, to impair its activation. The results demonstrated that treatment with XAV-939 significantly inhibited the CRP-1 overexpression-induced proliferation and migration of BEL-7405 cells (Fig. 5C and D).

Discussion

HCC is a highly prevalent type of tumor worldwide, thus seriously threatening human health. HCC is characterized by insidious onset, high malignancy, metastasis and recurrence rates, rapid progression and poor prognosis (25). Due to the poor understanding of the mechanism underlying HCC and its insidious nature, the early diagnosis and effective treatment of HCC are considered problematic. Therefore, the identification

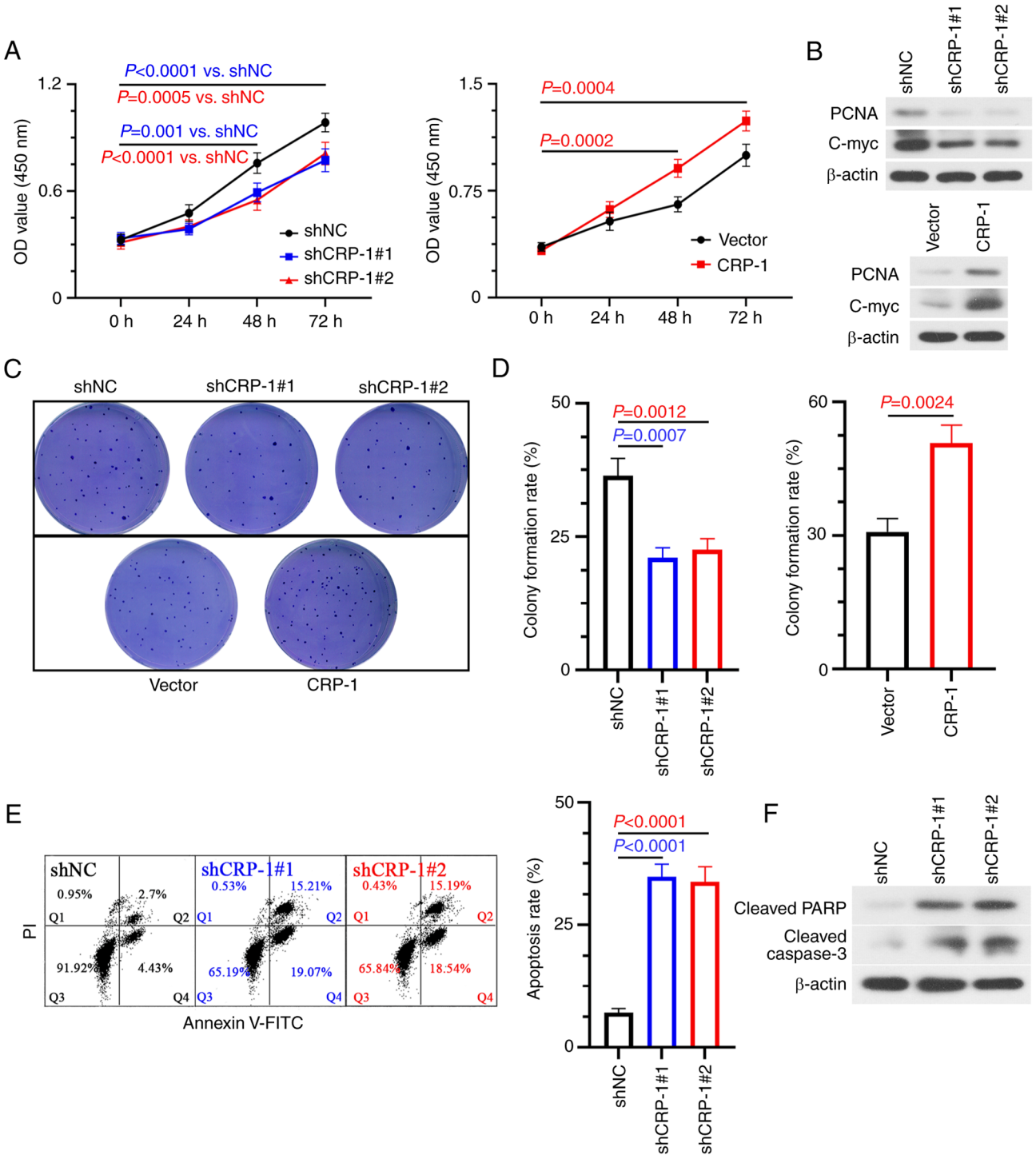

Figure 3. CRP-1 regulates the proliferation and apoptosis of hepatoma cells. (A) The proliferation ability of CRP-1-overexpressing BEL-7405 cells and that of CRP-1-depleted Hep 3B2.1-7 cells was quantitatively assessed using a Cell Counting Kit-8 assay. (B) The expression levels of proliferating cell nuclear antigen and c-Myc in CRP-1-overexpressing BEL-7405 and CRP-1-silencing Hep 3B2.1-7 cells were detected by western blot analysis. (C) Representative images of the formed colonies in CRP-1-overexpressing BEL-7405 cells, CRP-1-depleted Hep 3B2.1-7 cells and their corresponding control cells are presented. (D) Quantitative results of colony formation assay. (E) The percentage of apoptotic cells in CRP-1-depleted Hep 3B2.1-7 cells was determined by flow cytometry. (F) The expression levels of cleaved poly(ADP-ribose) polymerase and cleaved caspase 3 in CRP-1-depleted Hep 3B2.1-7 cells were determined using western blot analysis. Data are expressed as the mean ± SD of three independent experiments. CRP-1, cysteine rich protein 1.

of effective biomarkers for HCC has become a key event for its diagnosis and treatment. The current study verified that CRP-1 was upregulated in HCC cells and was involved in disease progression.

Cell proliferation and apoptosis are closely associated with HCC progression (26). Cell viability, colony formation and apoptosis assays were performed to investigate the effect of CRP-1 knockdown on the proliferation ability of HCC cells. Therefore, the results demonstrated that CRP-1 silencing significantly inhibited the survival and proliferation of HCC cells. In addition, the expression levels of PCNA and c-Myc, two cell cycle-related indicators of cell proliferation (27,28),

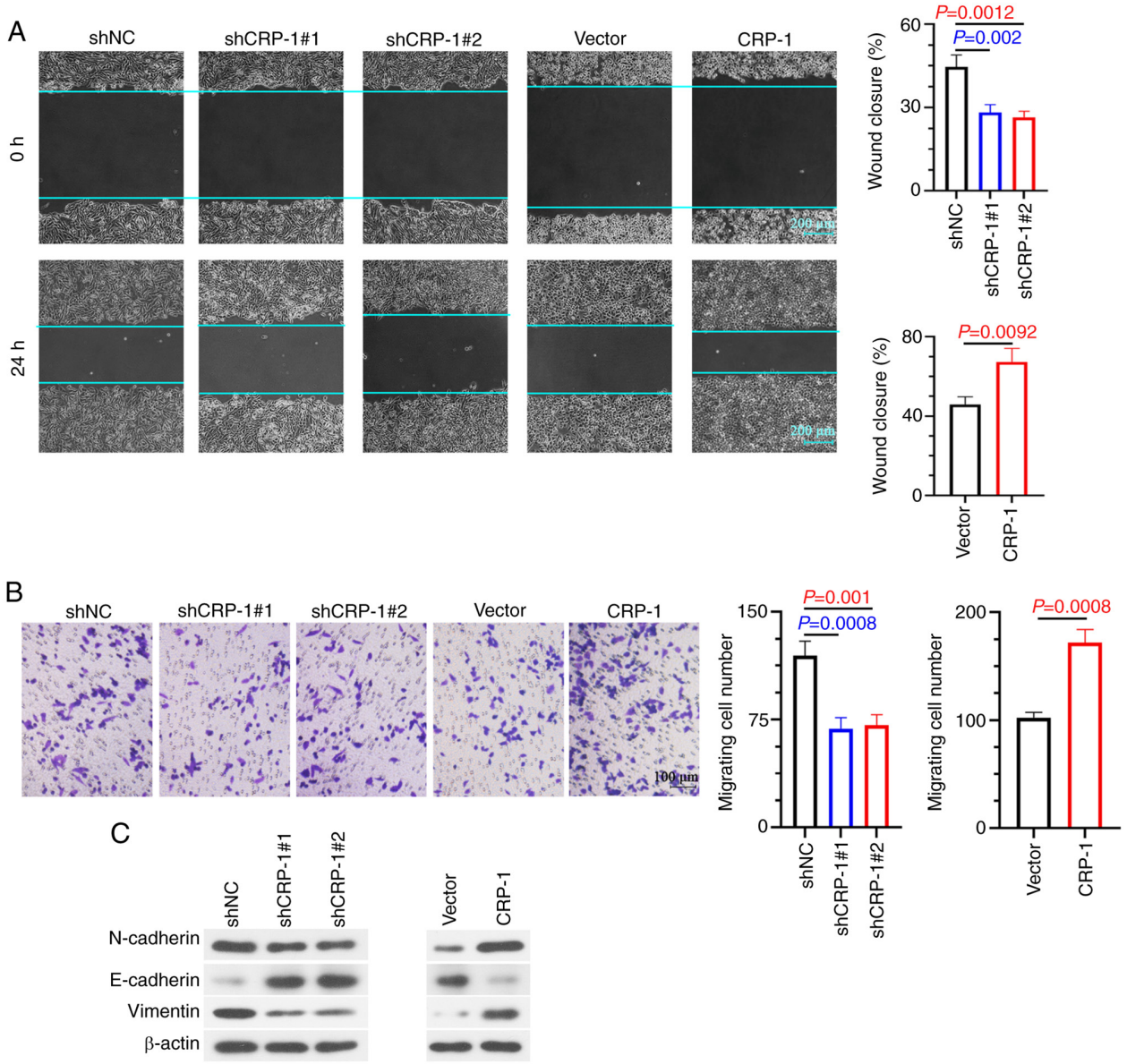

Figure 4. CRP-1 regulates the migration, invasion and epithelial-mesenchymal transition of hepatoma cells. (A) Wound healing assay showing the distance between wound edges in CRP-1-overexpressing BEL-7405 cells, CRP-1-depleted Hep 3B2.1-7 cells and their corresponding control cells at 24 h (scale bar=200 μm; original magnification 100x). The bar graph illustrates the quantification of the wound healing assay results. (B) Representative images of the Transwell migration assays (scale bar=100 μm; original magnification, 200x). The bar graph illustrates the quantification of the number of migrated cell. (C) The protein expression levels of N-cadherin, E-cadherin, and vimentin in CRP-1-overexpressing BEL-7405 and CRP-1-depleted Hep 3B2.1-7 cells were determined by western blo analysis. Data are expressed as the mean ± SD of three independent experiments. CRP-1, cysteine rich protein 1.

were reduced in CRP-1-depleted HCC cells, thus suggesting that CRP-1 could regulate the expression of PCNA and c-Myc to promote HCC cell proliferation. It has been reported that CRP-1 regulates the proliferation of cancer cells. Therefore, in thyroid cancer, CRP-1 silencing, which acts as a proto-oncogene, could inhibit the proliferation and induce the apoptosis of the thyroid cancer cell lines, SW579 and TT (17). The above findings were consistent with those observed in the present study. However, Latonen *et al* (29) showed that CRP-1 knockdown had no significant effect on tumor cell proliferation and apoptosis, thus supporting the cancer type-dependent function of CRP-1.

Apoptosis is a tightly controlled process, that is regulated by several gene, such as the members of the caspase family (30). In colorectal cancer, caspase 3 could trigger cell apoptosis induced by CRP-1 (31). Caspase 3, a key molecule involved in the induction of cancer cell apoptosis, is stimulated and converted in its active form, namely cleaved caspase 3, which is the dominant cleavage enzyme involved in promoting cell apoptosis (32,33). It has been also reported that during apoptosis, caspase 3 can cleave PARP, which is involved in DNA damage and repair, to induce apoptosis (34). Cleaved caspase 3 and cleaved PARP are two pivotal targets of the mitochondria-mediated apoptosis pathway (35). Herein, the results demonstrated that CRP-1 silencing downregulated cleaved caspase 3 and cleaved PARP in HCC cells, thus suggesting that CRP-1 induced apoptosis could be associated with the activation of the mitochondrial apoptotic pathway through caspase 3. However, whether CRP-1 regulates cell apoptosis only via the mitochondrial apoptotic pathway remains elusive. CRP-1

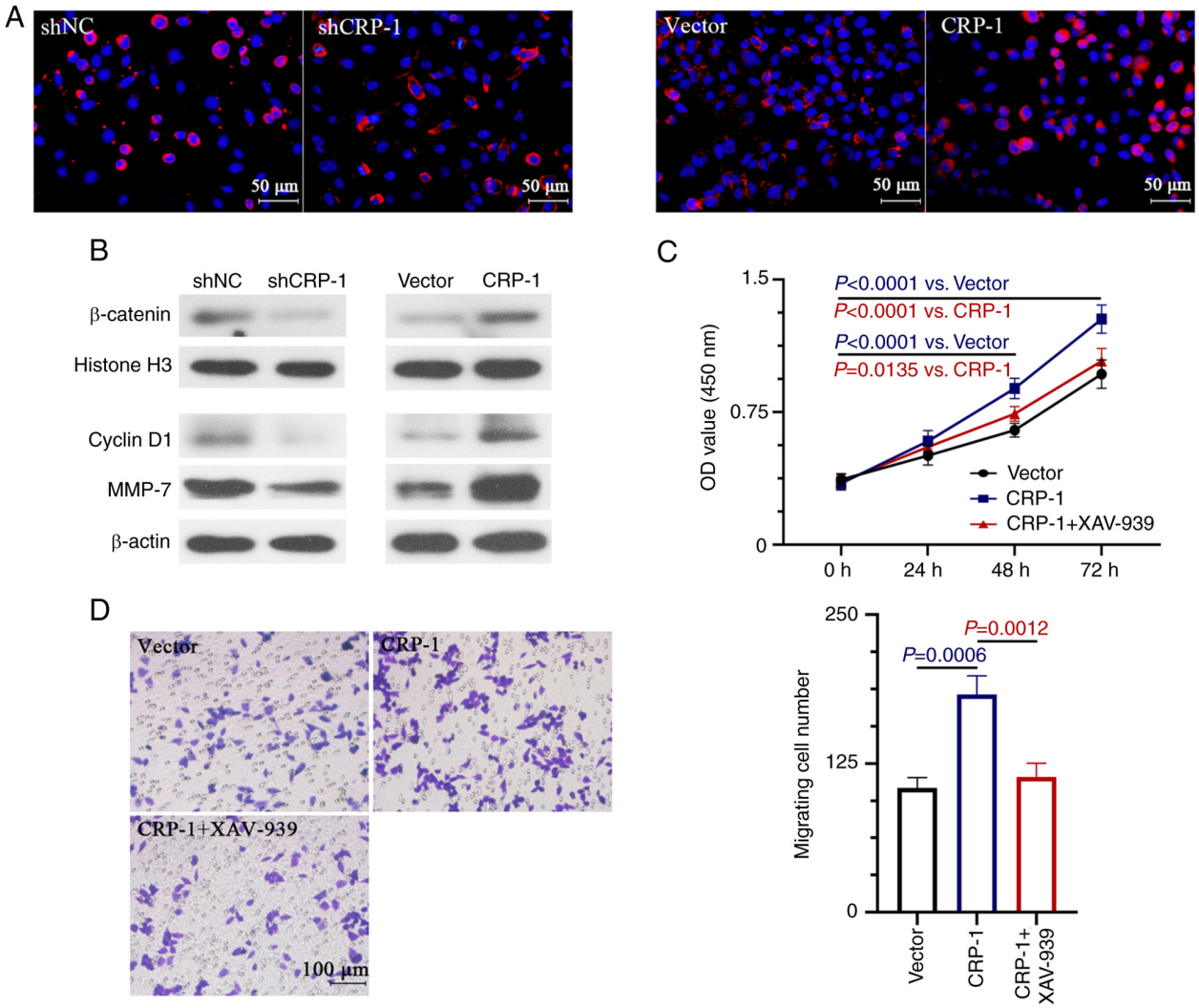

Figure 5. Effect of CRP-1 on the Wnt/β-catenin signal pathway. (A) Immunofluorescence staining of β-catenin in CRP-1-overexpressing BEL-7405 cells, CRP-1-depleted Hep 3B2.1-7 cells and their coresponding negative control cells (scale bar=50 μm; original magnification, 400x). Red dots indicate CRP-1 protein; blue indicates cell nuclei (4',6-diamidino-2-phenylindole). (B) The expression levels of β-catenin, cyclin D1, and matrix metalloproteinase-7 were detected in CRP-1-overexpressing BEL-7405 and CRP-1-depleted Hep 3B2.1-7 cells by western blot analysis. The effect of XAV-939, a Wnt/β-catenin inhibitor, on (C) cell viability and (D migration (scale bar=100 μm; original magnification, 200x) is shown. Data are expressed as the mean ± SD of three independent experiments. CRP-1, cysteine rich protein 1.

is a zinc finger protein that directs protein-protein interactions in the presence of cysteine (11). A previous study revealed that CRP-1 could interact with Fas to mediate its degradation to promote colorectal cancer cell apoptosis (31). Fas is a significant mediator of the death receptor-dependent apoptotic pathway, another key pathway involved in the regulation of apoptosis (36). The aforementioned studies revealed another possible mechanism underlying CRP-1 induced apoptosis. This mechanism could be explored in HCC in a follow-up study.

Due to its high migration rate, HCC has the highest recurrence rate among human cancer types (37). Several studies have suggested that EMT serves a key role in tumor metastasis through tumor cell invasion and distant organ diffusion (38,39). Therefore, a previous study demonstrated that CRP-1 knockdown inhibited the EMT of SW620 and LoVo cells, thereby attenuating the invasion and migration ability of colorectal cancer cells (40). Based on the above findings, the present study aimed to validate the association between CRP-1 and EMT in HCC. The results showed that CRP-1 silencing upregulated E-cadherin and downregulated N-cadherin and vimentin. EMT is characterized by the transformation of epithelial cells into mesenchymal cells (41). E-cadherin, an epithelial marker, is a cell adhesion molecule that anchors epithelial cells via linking catenins to the cell cytoskeleton. The expression of E-cadherin renders cancer cells incapable of metastasis, thus acquiring a low-invasive phenotype (42). N-cadherin and vimentin are two mesenchymal markers. Therefore, a previous study showed that N-cadherin and vimentin upregulation could promote the migration or metastasis of tumor cells to different organs (43). Herein, the results also revealed that CRP-1 knockdown inhibited the EMT-induced invasion and migration of HCC cells.

In a recent study on ovarian cancer, Kyoto Encyclopedia of Genes and Genomes analysis revealed that CRP-1 was enriched in the term 'Wnt/β-catenin signaling pathway'. The above finding was verified by western blot analysis, showing that CRP-1 activated the Wnt/β-catenin signaling pathway to affect EMT (44). It has been reported that Wnt/β-catenin

signaling plays a key role in tumor development, including HCC (45,46). Catenin, which is transferred from the cytoplasm to the nucleus when activated, also serves a critical role in this pathway. Upon entry into the nucleus, β-catenin binds to different transcription factors to activate its downstream target genes, such as cyclinD1 and MMP-7 (47), which in turn affect cell proliferation, invasion and migration. Cyclin D1 is a significant protein involved in the regulation of the cell cycle. When cyclin D1 is upregulated, it accelerates tumor cell proliferation and induces tumor formation. A study demonstrated that cyclin D downregulation could promote the accumulation of cells to the G1 phase of the cell cycle, thus suppressing cell invasion and metastasis (48). MMP-7 is a proteolytic enzyme closely associated with tumor cell invasion and metastasis (49). It can degrade the extracellular matrix, which is a significant factor involved in malignant tumor cell invasion and metastasis. The present study demonstrated that CRP-1 knockdown could block the activation of the Wnt/β-catenin signaling pathway, which could be involved not only in the regulation of EMT, but also in cell proliferation. It has been reported that c-Myc can affect cell proliferation. However, c-Myc is also considered as a significant downstream target gene of the Wnt/β-catenin signaling pathway (50). Herein, c-Myc was downregulated in CRP-1-depleted HCC cells. Subsequently, to further explore whether CRP-1 could affect the growth and metastasis of HCC cells through Wnt/β-catenin signaling, cells were treated with XAV-939, a Wnt/β-catenin signaling inhibitor. The results revealed that cell treatment with XAV-939 significantly attenuated the CRP-1 overexpression-induced proliferation and migration of HCC cells. Consistently, a previous study also showed that CRP-1 could promote cell migration, invasion and EMT via activating the Wnt/β-catenin signaling pathway (51).

In conclusion the results of the current study demonstrated that CRP-1 was upregulated in HCC, while CRP-1 knockdown inhibited the growth and metastasis of HCC cells via suppressing the Wnt/β-catenin signal pathway. Overall, CRP-1 could be considered as a potential prognostic biomarker and therapeutic target of HCC.

Acknowledgements

Not applicable.

Funding

This work was supported by the Shaanxi Natural Science Basic Research Program (grant no. 2020JM-337) and the Tangdu Hospital Science and Technology Innovation Development Fund (grant no. 2017JSYJ007).

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

SL, XD, KT and XH conceived the study. SL, KT, XH, YZ and SZ performed the experiments and analyzed the data. KT performed the bioinformatics analysis. ZY and GD helped to perform the experiments and check the results. SL drafted the original manuscript. SL and XD confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Chan HL and Sung JJ: Hepatocellular carcinoma and hepatitis B virus. Semin Liver Dis 26: 153-161, 2006.
2. Parkin DM, Bray F, Ferlay J and Pisani P: Global cancer statistics, 2002. CA Cancer J Clin 55: 74-108, 2005.
3. Ringelhan M, Pfister D, O'Connor T, Pikarsky E and Heikenwalder M: The immunology of hepatocellular carcinoma. Nat Immunol 19: 222-232, 2018.
4. Petrick JL, Braunlin M, Laversanne M, Valery PC, Bray F and McGlynn KA: International trends in liver cancer incidence, overall and by histologic subtype, 1978-2007. Int J Cancer 139: 1534-1545, 2016.
5. Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ and He J: Cancer statistics in China, 2015. CA Cancer J Clin 66: 115-132, 2016.
6. Allemani C, Matsuda T, Di Carlo V, Harewood R, Matz M, Nikšić M, Bonaventure A, Valkov M, Johnson CJ, Estève J, *et al*: Global surveillance of trends in cancer survival 2000-14 (CONCORD-3): Analysis of individual records for 37 513 025 patients diagnosed with one of 18 cancers from 322 population-based registries in 71 countries. Lancet 391: 1023-1075, 2018.
7. Finn RS: Emerging targeted strategies in advanced hepatocellular carcinoma. Semin Liver Dis 33 (Suppl 1): S11-19, 2013.
8. Lohitesh K, Chowdhury R and Mukherjee S: Resistance a major hindrance to chemotherapy in hepatocellular carcinoma: An insight. Cancer Cell Int 18: 44, 2018.
9. Daher S, Massarwa M, Benson AA and Khoury T: Current and future treatment of hepatocellular carcinoma: An updated comprehensive review. J Clin Transl Hepatol 6: 69-78, 2018.
10. Jurata LW, Kenny DA and Gill GN: Nuclear LIM interactor, a rhombotin and LIM homeodomain interacting protein, is expressed early in neuronal development. Proc Natl Acad Sci USA 93: 11693-11698, 1996.
11. Khoo C, Blanchard RK, Sullivan VK and Cousins RJ: Human cysteine-rich intestinal protein: cDNA cloning and expression of recombinant protein and identification in human peripheral blood mononuclear cells. Protein Expr Purif 9: 379-387, 1997.
12. Fernandes PR, Samuelson DA, Clark WR and Cousins RJ: Immunohistochemical localization of cysteine-rich intestinal protein in rat small intestine. Am J Physiol 272: G751-G759, 1997.
13. Cousins RJ and Lanningham-Foster L: Regulation of cysteine-rich intestinal protein, a zinc finger protein, by mediators of the immune response. J Infect Dis 182 (Suppl 1): S81-S84, 2000.
14. Baumhoer D, Elsner M, Smida J, Zillmer S, Rauser S, Schoene C, Balluff B, Bielack S, Jundt G, Walch A and Nathrath M: CRIP1 expression is correlated with a favorable outcome and less metastases in osteosarcoma patients. Oncotarget 2: 970-975, 2011.
15. Ludyga N, Englert S, Pflieger K, Rauser S, Braselmann H, Walch A, Auer G, Höfler H and Aubele M: The impact of cysteine-rich intestinal protein 1 (CRIP1) in human breast cancer. Mol Cancer 12: 28, 2013.
16. Lambropoulou M, Deftereou TE, Kynigopoulos S, Patsias A, Anagnostopoulos C, Alexiadis G, Kotini A, Tsaroucha A, Nikolaidou C, Kiziridou A, *et al*: Co-expression of galectin-3 and CRIP-1 in endometrial cancer: Prognostic value and patient survival. Med Oncol 33: 8, 2016.

17. Li HG, Zhao LH, Zhang ZH, Liu JZ, Ren K, Li SY and Su ZJ: The impact of cysteine-rich intestinal protein 1 (CRIP1) on thyroid carcinoma. Cell Physiol Biochem 43: 2037-2046, 2017.
18. Wang Q, Williamson M, Bott S, Brookman-Amissah N, Freeman A, Nariculam J, Hubank MJF, Ahmed A and Masters JR: Hypomethylation of WNT5A, CRIP1 and S100P in prostate cancer. Oncogene 26: 6560-6565, 2007.
19. Terris B, Blaveri E, Crnogorac-Jurcevic T, Jones M, Missiaglia E, Ruszniewski P, Sauvanet A and Lemoine NR: Characterization of gene expression profiles in intraductal papillary-mucinous tumors of the pancreas. Am J Pathol 160: 1745-1754, 2002.
20. Groene J, Mansmann U, Meister R, Staub E, Roepcke S, Heinze M, Klaman I, Brümmendorf T, Hermann K, Loddenkemper C, *et al*: Transcriptional census of 36 microdissected colorectal cancers yields a gene signature to distinguish UICC II and III. Int J Cancer 119: 1829-1836, 2006.
21. He G, Zou L, Zhou L, Gao P, Qian X and Cui J: Cysteine-rich intestinal protein 1 silencing inhibits migration and invasion in human colorectal cancer. Cell Physiol Biochem 44: 897-906, 2017.
22. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
23. Matthews JM, Lester K, Joseph S and Curtis DJ: LIM-domain-only proteins in cancer. Nat Rev Cancer 13: 111-122, 2013.
24. Vilchez V, Turcios L, Marti F and Gedaly R: Targeting Wnt/β-catenin pathway in hepatocellular carcinoma treatment. World J Gastroenterol 22: 823-832, 2016.
25. Forner A, Llovet JM and Bruix J: Hepatocellular carcinoma. Lancet 379: 1245-1255, 2012.
26. Chisari FV, Klopchin K, Moriyama T, Pasquinelli C, Dunsford HA, Sell S, Pinkert CA, Brinster RL and Palmiter RD: Molecular pathogenesis of hepatocellular carcinoma in hepatitis B virus transgenic mice. Cell 59: 1145-1156, 1989.
27. Grandori C, Cowley SM, James LP and Eisenman RN: The Myc/Max/Mad network and the transcriptional control of cell behavior. Annu Rev Cell Dev Biol 16: 653-699, 2000.
28. Cole MD: The myc oncogene: Its role in transformation and differentiation. Annu Rev Genet 20: 361-384, 1986.
29. Latonen L, Järvinen PM and Laiho M: Cytoskeleton-interacting LIM-domain protein CRP1 suppresses cell proliferation and protects from stress-induced cell death. Exp Cell Res 314: 738-747, 2008.
30. Debatin KM: Apoptosis pathways in cancer and cancer therapy. Cancer Immunol Immunother 53: 153-159, 2004.
31. Zhang L, Zhou R, Zhang W, Yao X, Li W, Xu L, Sun X and Zhao L: Cysteine-rich intestinal protein 1 suppresses apoptosis and chemosensitivity to 5-fluorouracil in colorectal cancer through ubiquitin-mediated Fas degradation. J Exp Clin Cancer Res 38: 120, 2019.
32. Medina V, Edmonds B, Young GP, James R, Appleton S and Zalewski PD: Induction of caspase-3 protease activity and apoptosis by butyrate and trichostatin A (inhibitors of histone deacetylase): Dependence on protein synthesis and synergy with a mitochondrial/cytochrome c-dependent pathway. Cancer Res 57: 3697-3707, 1997.
33. Asselin E, Mills GB and Tsang BK: XIAP regulates Akt activity and caspase-3-dependent cleavage during cisplatin-induced apoptosis in human ovarian epithelial cancer cells. Cancer Res 61: 1862-1868, 2001.
34. Affar EB, Germain M, Winstall E, Vodenicharov M, Shah RG, Salvesen GS and Poirier GG: Caspase-3-mediated processing of poly(ADP-ribose) glycohydrolase during apoptosis. J Biol Chem 276: 2935-2942, 2001.
35. Sivalingam KS, Paramasivan P, Weng CF and Viswanadha VP: Neferine potentiates the antitumor effect of cisplatin in human lung adenocarcinoma cells via a mitochondria-mediated apoptosis pathway. J Cell Biochem 118: 2865-2876, 2017.
36. Strasser A, O'Connor L and Dixit VM: Apoptosis signaling. Annu Rev Biochem 69: 217-245, 2000.
37. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J and Jemal A: Global cancer statistics, 2012. CA Cancer J Clin 65: 87-108, 2015.
38. Aiello NM and Kang Y: Context-dependent EMT programs in cancer metastasis. J Exp Med 216: 1016-1026, 2019.
39. Heerboth S, Housman G, Leary M, Longacre M, Byler S, Lapinska K, Willbanks A and Sarkar S: EMT and tumor metastasis. Clin Transl Med 4: 6, 2015.
40. He G, Zhu H, Yao Y, Chai H, Wang Y, Zhao W, Fu S and Wang Y: Cysteine-rich intestinal protein 1 silencing alleviates the migration and invasive capability enhancement induced by excessive zinc supplementation in colorectal cancer cells. Am J Transl Res 11: 3578-3588, 2019.
41. Lamouille S, Xu J and Derynck R: Molecular mechanisms of epithelial-mesenchymal transition. Nat Rev Mol Cell Biol 15: 178-196, 2014.
42. Mendonsa AM, Na TY and Gumbiner BM: E-cadherin in contact inhibition and cancer. Oncogene 37: 4769-4780, 2018.
43. Odero-Marah V, Hawsawi O, Henderson V and Sweeney J: Epithelial-mesenchymal transition (EMT) and prostate cancer. Adv Exp Med Biol 1095: 101-110, 2018.
44. Liu Y, Li W, Luo J, Wu Y, Xu Y, Chen T, Zhang W and Fu F: Cysteine-rich intestinal protein 1 served as an epithelial ovarian cancer marker via promoting Wnt/β-catenin-mediated EMT and tumour metastasis. Dis Markers 2021: 3566749, 2021.
45. Onyido EK, Sweeney E and Nateri AS: Wnt-signalling pathways and microRNAs network in carcinogenesis: Experimental and bioinformatics approaches. Mol Cancer 15: 56, 2016.
46. He S and Tang S: WNT/β-catenin signaling in the development of liver cancers. Biomed Pharmacother 132: 110851, 2020.
47. Li Q, Sun M, Wang M, Feng M, Yang F, Li L, Zhao J, Chang C, Dong H, Xie T and Chen J: Dysregulation of Wnt/β-catenin signaling by protein kinases in hepatocellular carcinoma and its therapeutic application. Cancer Sci 112: 1695-1706, 2021.
48. Kim CJ, Terado T, Tambe Y, Mukaisho KI, Sugihara H, Kawauchi A and Inoue H: Anti-oncogenic activities of cyclin D1b siRNA on human bladder cancer cells via induction of apoptosis and suppression of cancer cell stemness and invasiveness. Int J Oncol 52: 231-240, 2018.
49. Taniguchi M, Matsuura K, Nakamura R, Kojima A, Konishi M and Akizawa T: MMP-7 cleaves amyloid β fragment peptides and copper ion inhibits the degradation. Biometals 30: 797-807, 2017.
50. Baarsma HA, Königshoff M and Gosens R: The WNT signaling pathway from ligand secretion to gene transcription: Molecular mechanisms and pharmacological targets. Pharmacol Ther 138: 66-83, 2013.
51. Zhang LZ, Huang LY, Huang AL, Liu JX and Yang F: CRIP1 promotes cell migration, invasion and epithelial-mesenchymal transition of cervical cancer by activating the Wnt/β-catenin signaling pathway. Life Sci 207: 420-427, 2018.

This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.