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HDGF-related protein-2 (HRP-2) acts as an oncogene to promote cell growth in hepatocellular carcinoma

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ABSTRACT

HDGFRP2 (HRP-2) belongs to the Hepatoma-derived growth factor (HDGF)-related proteins (HRPs) family, which are characterized by a conserved HATH/PWWP domain at a well-conserved region of the N-terminus. However, the cellular function of HRP-2 remains unknown. In this study, we showed for the first time that HRP-2 is frequently overexpressed in human HCC tissues at mRNA and protein levels. We further showed that HRP-2 can promote HCC cells growth in vitro and xenograft tumors in vivo. Using protein affinity purification methods, we searched for functional partners of HRP-2, and found that HRP-2 interacts with various proteins known to be involved in transcription elongation and processing. Furthermore, we demonstrate HRP-2 interacts and co-localizes with RNA processing regulator IWS1, and positively regulated the mRNA level of Cyclin D1. Together, our study suggests HRP-2 may act as an mRNA processing co-factor to promote cells growth by regulating the mRNA of key oncogenes, which can be explored further for cancer treatment.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer death worldwide and the fourth most prevalent malignant disease among adults in East Asia and sub-Saharan Africa [1]. Although many signaling pathways have been implicated in HCC, the molecular basis of liver carcinogenesis remains largely unknown.

HDGF-related protein-2 (HRP-2) belongs to the family of hepatoma-derived growth factor (HDGF)-related proteins (HRPs), which also includes HDGF, HDGFL1, HRP-3, and LEDGF [2]. All family members share a conserved NH2-terminal HATH/PWWP domain at a well-conserved region of the N-terminus. HDGF is the founding member of this family, and has been most extensively studied [2]. It was originally purified from conditional media of the human hepatoma cell line Huh-7 and was shown to be capable of stimulating proliferation of Swiss 3T3 cells [3,4]. Many studies reported HDGF was overexpressed and played an important role in the development and progression of various types of human cancers [5–7]. Our recent studies also showed another HRPs member, HRP-3 was highly upregulated in HCC tissues. HRP-3 can promote anchorage-dependent and -independent growth of HCC cells in vitro and xenograft tumor growth in vivo, possibly through activating the MAPK-ERK pathway [8]. In contrast to HDGF (~240aa) or HRP-3 (~212aa), HRP-2 encodes a larger protein (~671aa), and has not been characterized in previous studies. The cellular functions, physiological and pathological relevance of HRP-2 remain totally unknown.

In the present study, we demonstrated that the HRP-2 is frequently overexpressed in human HCC tissues and required for HCC cells growth in vitro and in vivo. Moreover, we demonstrated that HRP-2 interacts with mRNA processing regulator IWS1. Lastly, we showed that HRP-2 and IWS1 cooperatively regulate the mRNA and protein level of Cyclin D1, which may explain the tumor-promoting functions of HRP-2.

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2. Materials and methods

2.1. Cell culture and transfection

The 293T cells were obtained from American Type Culture Collection (ATCC, USA). Human HCC cell lines SMMC-7721, QGY-7703 were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). All cells were cultured in DMEM with 10% (vol/vol) fetal bovine serum (FBS), at 37 °C in a humidified incubator with 5% CO_2. Cells were transiently transfected using Lipofectamine 2000 or 3000 (Invitrogen, USA) according to manufacturer's instructions.

2.2. Human tissue samples

Surgical resection specimens were obtained from Qidong Liver Cancer Institute (Jiangsu province, China), or Eastern Hepatobiliary Surgery Hospital (Shanghai, China). The tumor specimens were immediately snap-frozen in liquid nitrogen and stored at −80 °C for further analysis. Informed consent was obtained from each subject or subject's guardian after approval by the appropriate hospital Ethics Committee.

2.3. Expression constructs

The full length of HRP-2 and IWS1 cDNAs were amplified from Human liver Marathon cDNA library (Clontech, USA), and subcloned into pCIN4-FLAG–HA, pCMV-HA and pcDNA3.1-Myc expression vectors.

2.4. RNA interference

The siRNA oligos were purchased from Genepharma Inc (Shanghai). The siRNA oligos sequences for HRP-2 are: si-HRP-2#1: 5′- GCCCAACAAGAGGAAAGGC-3′; si-HRP-2#2: 5′-GCUGCACAGUGAGAUCAAG-3′. The siRNA oligos sequences for IWS1 are: si-IWS1#1: 5′-GGAUGAUGUAAAUGAGCAA-3′. si-IWS1#2: 5′- GGACAGCGACTCTGAATCT-3′. The sequence of negative control is: si-Control: 5′-ACAGACUUCGGAGUACCUG-3′.

2.5. Antibodies

Generation of affinity-purified polyclonal antibody against HRP-2 or IWS1 was performed according to the reference with some modifications. The antibodies were raised in rabbits using GST-HRP-2 (450–671aa) and IWS1 (600–819aa) fusion proteins as the immuogens. The following commercial antibodies were used: Cyclin B1 (ab32053; Abcam), Cyclin D1 (ab134175; Abcam), Cyclin E1 (ab33911; Abcam), CDK1 (ab133327; Abcam), CDK2 (ab32147; Abcam), p21 (ab109520; Abcam), p27 (ab32034; Abcam), FLAG (M2; Sigma), Myc (9E10; Sigma), HA (MM5-101R; Convance), Actin (AC-74; Sigma).

2.6. Northern blot

Total RNA was extracted from tissues using Trizol reagent, denatured with formaldehyde, and blotted onto nylon membranes. The probe for HRP-2 was labeled with [α-32P]dCTP using a Megaprime DNA labeling kit (GE Healthcare, UK) and used for hybridization.

2.7. Immunoprecipitation

To immunoprecipitate the ectopically expressed FLAG-tagged proteins, transfected cells were lysed 24 h post-transfection in BC100 buffer. The whole-cell lysates were immunoprecipitated with the monoclonal anti-FLAG antibody-conjugated M2 agarose beads (Sigma, USA) at 4 °C overnight. After three washes with FLAG lysis buffer, followed by two washes with BC100 buffer, the bound proteins were eluted using FLAG-Peptide (Sigma, USA)/BC100 for 3 h at 4 °C. The eluted material was resolved by SDS-PAGE. To immunoprecipitate the endogenous proteins, cells were lysed with 1 × cell lysis buffer (Cell Signaling, USA), and the lysate was centrifuged. The supernatant was precleared with protein A/G beads (Sigma, USA) and incubated with indicated antibody overnight. Thereafter, protein A/G beads were applied, all at 4 °C. After 2 h of incubation, pellets were washed five times with lysis buffer and resuspended in sample buffer and analyzed by SDS-PAGE.

2.8. Western blot

Cell lysates and immunoprecipitates were subjected to SDS-PAGE and proteins were transferred to nitrocellulose membranes (GE Healthcare, USA). The membrane was blocked in PBS containing 5% non-fat milk and 0.1% Tween-20, washed twice in PBS, and incubated with primary antibody at room temperature for 2 h, followed by incubation with secondary antibody at room temperature for 45 min. Afterward, the proteins of interest were visualized using ECL chemiluminescence system (Santa Cruz, USA).

2.9. Quantitative RT-PCR

Total RNA was isolated from transiently transfected cells using the TRIzol reagent (Tiangen, China), and cDNA was reversed-transcribed using the Superscript RT kit (TOYOBO, Japan), according to the manufacturer's instructions. PCR amplification was performed using the SYBR Green PCR master mix Kit (TOYOBO, Japan). The Primers of Cyclin D1 are: Cyclin D1-F: 5′-GAACACGGCTCACGCTTACCTC- 3′, Cyclin D1-R: 5′- ACTTGTGCCC TTGCCCCATC-3′, All quantization were normalized to the level of endogenous control GAPDH.

2.10. Cell proliferation assay

Cell proliferation rate was determined using Cell Counting Kit-8 (CCK-8) according to the manufacturer's protocol (Dojindo Laboratories, Japan). Briefly, The Ishikawa cells were seeded onto 96-well plates at a density of 2000 cells per well. During a 2 to 7-d culture periods, 10 μl of the CCK-8 solution was added to cell culture, and incubated for 2 h. The resulting color was assayed at 450 nm using a microplate absorbance reader (Bio-Rad, USA). Each assay was carried out in triplicate.

2.11. Statistical analysis

The data in this study were expressed as the mean ± S.D. from three independent experiments. Statistical analysis was performed using one-way ANOVA with a Student's t-test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. HRP-2 is overexpressed in hepatocellular carcinoma

To explore whether the expression level of HRP-2 was altered in human HCCs like other HRPs (HDGF or HRP-3), we first examined the expression status of the HRP-2 in 16 different human tissues by Northern blot. Northern blot analysis demonstrated that HRP-2 mRNA, at approximately 2.4 kb, are predominantly expressed in the heart, skeletal muscle, Ovary and testis, with low levels in the

liver and other tissues (Fig. 1A). Northern blot analysis further showed that HRP-2 mRNA was expressed in 12 paired HCC and adjacent non-cancerous tissues. Strikingly, the mRNA level of HRP-2 is much higher in 9 out of 12 HCCs compared with adjacent non-cancerous tissues (Fig. 1B). Next, we extended our analysis by examining the mRNA level of HRP-2 in a large cohort by quantitative RT-PCR methods. Fig. 1C showed the log2-transformed fold changes of HRP-2 mRNA expression ratio of T/N (cancer tissues/non-cancerous tissues), and a 2-fold threshold was set for significant changes in expression. As shown in Fig. 1C, the expression of HRP-2 was significantly elevated in 21 of 45 (46.7%) cases compared with adjacent non-cancerous tissues. 21 of 45 (46.6%) cases showed no significant alteration, and only 3 of 45 (6.7%) cases showed slight down-regulation of HRP-2 in HCC. This results revealed HRP-2 expression was elevated in a large proportion of HCC tissues (Fig. 1C, $p < 0.001$). Moreover, statistical analysis revealed that HRP-2 overexpression was correlated with larger tumor size ($p = 0.0072$) and Tumor encapsulation ($p = 0.031$) (Table. S1). In agreement with the increased expression of HRP-2 mRNA in HCCs, HRP-2 protein expression was also upregulated in 15 out of 17 HCCs as assessed by western blot (Fig. 1D). Collectively, we demonstrated by various approaches that HRP-2 mRNA and protein are frequently overexpressed in human HCC tissues.

3.2. HRP-2 promotes HCC cells growth in vitro and in vivo

Since HRP-2 was frequently overexpressed in HCCs, we want determine whether HRP-2 enhance HCC cell growth like HDGF or

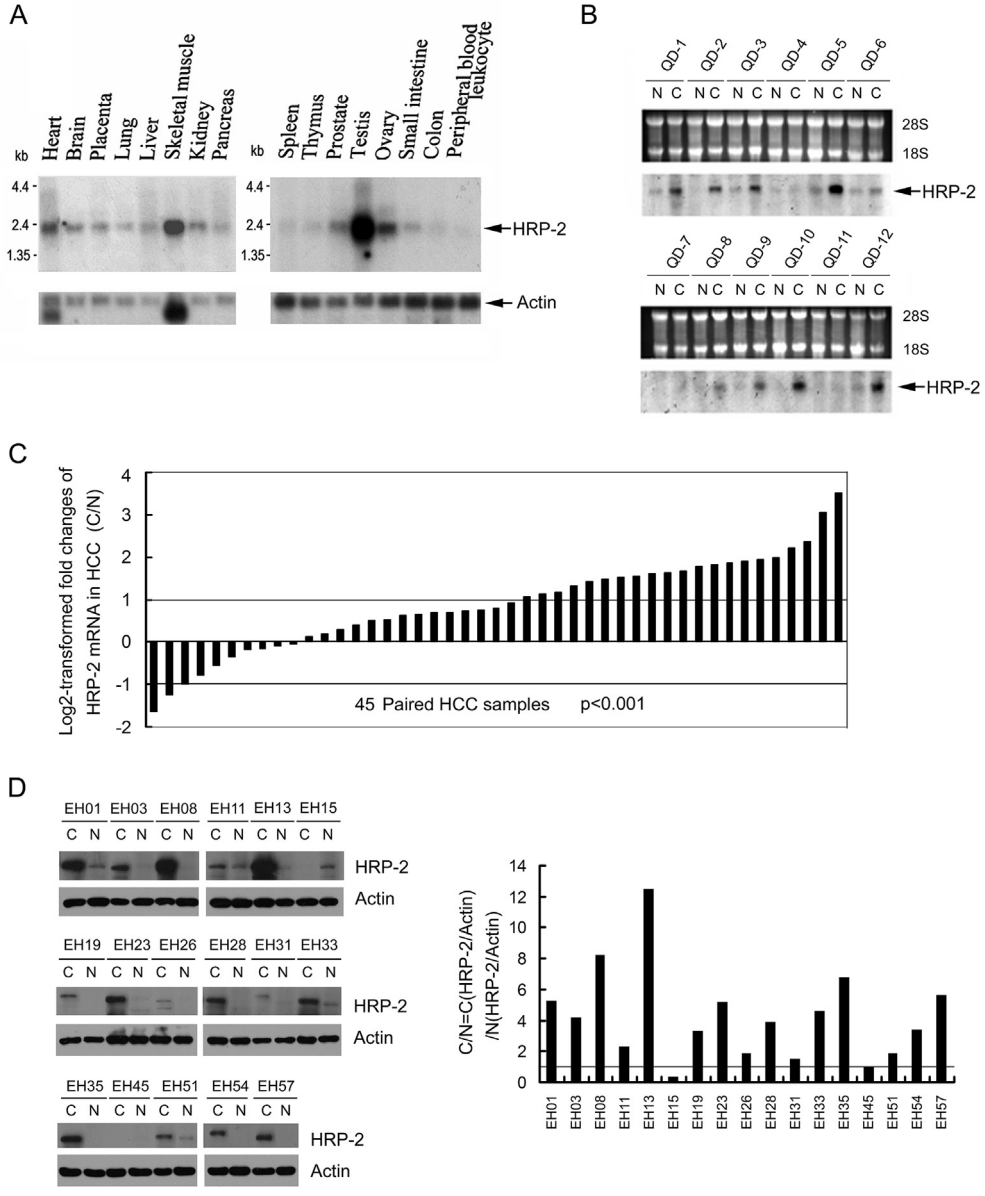

Fig. 1. HRP-2 was overexressed in human HCCs. (A) Northern blot analysis of HRP-3 mRNA expression in 16 human adult tissues. Arrow, HRP-2 transcript. Expression of the human β−actin served as a loading control. (B) The mRNA level of HRP-2 was analyzed in 12 paired HCC with their corresponding non-cancerous tissues by Northern blot. C; cancer, N; non-cancerous tissues. (D) The mRNA level of HRP-2 was analyzed in 45 paired HCC with their corresponding non-cancerous tissues by Quantitative RT-PCR. Log2-transformed fold changes of HRP-2 mRNA with respect to non-cancerous specimens were normalized to GAPDH. (C) The protein level of HRP-2 was analyzed in additional 12 paired HCC and their corresponding non-cancerous specimens by Western blot with HRP-3 antibody. Fold change of HRP-2 protein with respect to non-cancerous specimens were normalized to β-Actin. The quantification of immunoblots is shown in the left panel.

HRP-3. We first used a loss-of-function approach to assess the role of HRP-2 in HCC cell growth. Two HRP-2 gene-specific siRNAs were designed. The HRP-2 protein level was substantially reduced in QGY-7703 cells transfected with siRNA si-HRP-2#1 and si-HRP-2#2, as compared with si-Control siRNAs (Fig. 2A, left panel). Moreover, knockdown of HRP-2 by siRNAs markedly suppressed QGY-7703 cells growth when compared with control knockdown cells (Fig. 2A, left panel). Similar results were obtained in SMMC-7721 cells transfected with HRP-2 siRNAs (Fig. 2A, right panel). To assess the effect of HRP-2 overexpression on growth of HCC cells, we established QGY-7703 cell lines that were stably transfected with control constructs, or pcDNA3.1-Myc-HRP-2, respectively. We measured the cells growth of QGY-7703 stable cell lines. Two independent Myc-HRP-2 overexpression clones were used in this study. As shown in Fig. 2B (left panel), HRP-2/QGY-7703 cells grew much faster than the control Vector/QGY-7703 cells. Similar results were obtained in SMMC-7721 cells stably expressing Myc-HRP-2 (Fig. 2B, right panel). The finding that HRP-2 promotes growth of HCC cells in vitro prompted us to determine whether it exerts a similar effect in vivo. Control or HRP-2 overexpression clone of QGY-7703 cells were injected subcutaneously into nude mice. The tumors were dissected from nude mice 28 days after injection. As shown in Fig. 2C, stably overexpression of HRP-2 markedly enhanced growth of QGY-7703 tumors in mice. Taken together, these results suggested that HRP-2 promotes HCC cell growth in vitro and in vivo.

3.3. HRP-2 interacts with IWS1

HRP-2 is an uncharacterized protein, and no interaction partners were reported in previous studies. Moreover, HRP-2 was predominantly localized to the nucleus and can't be secreted from cells as a growth factor, like HDGF or HRP-3 (data not shown).

In an attempt to elucidate HRP-2-mediated cellular function further, we established 293T derivative cell line stably expressing a FLAG-HA double tagged version of HRP-2. Tandem affinity purification with M2-agarose beads and HA-agarose beads followed by mass spectrometry analysis allowed us to discover several proteins that interacted with HRP-2 (Fig. 3A). Interestingly, majority of the potential HRP-2-interacting proteins were connected to gene transcription and mRNA metabolism, processing, and splicing (Table S1).

One of the high-confident HRP-2 interactors is IWS1, which showed most number of unique peptides in Mass Spectrometry results (Table. S2). IWS1 is known to contribute to the assembly of a transcriptional elongation complex on the Ser2-phosphorylated CTD of the large subunit of RNA polymerase II by interaction with transcription elongation factor Spt6 and histone H3 trimethyl

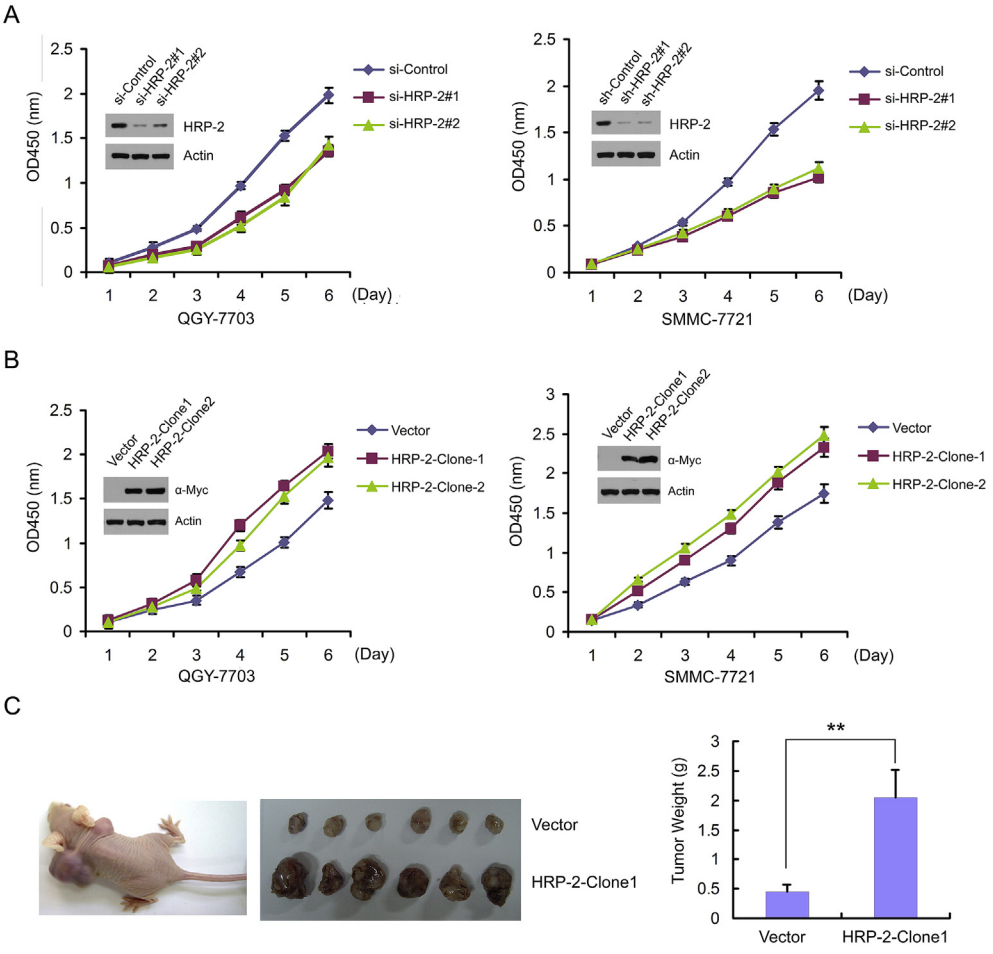

Fig. 2. HRP-2 promotes HCC cells growth. (A) QGY-7703 (left) or SMMC-7721 (right) cells were transfected with control or two HRP-2-specific siRNAs. After 24 h, the cell proliferation was measured at indicated days. (B) The cell growth of QGY-7703 (left panel) or SMMC-7721 (right panel) that stably transfected with control or pcDNA3.1-Myc-HRP-2 constructs were measured by CCK8 assay at indicated days. Two independent Myc-HRP-2 clones were used. (C) Xenografts were established by injecting s.c. QGY-7703/HRP-2 and QGY-7703/Vec cells to the left and right flanks of nude mice, respectively. The Mice and dissected tumors were photographed at 28 days after injection. Weight of dissected tumors was shown.

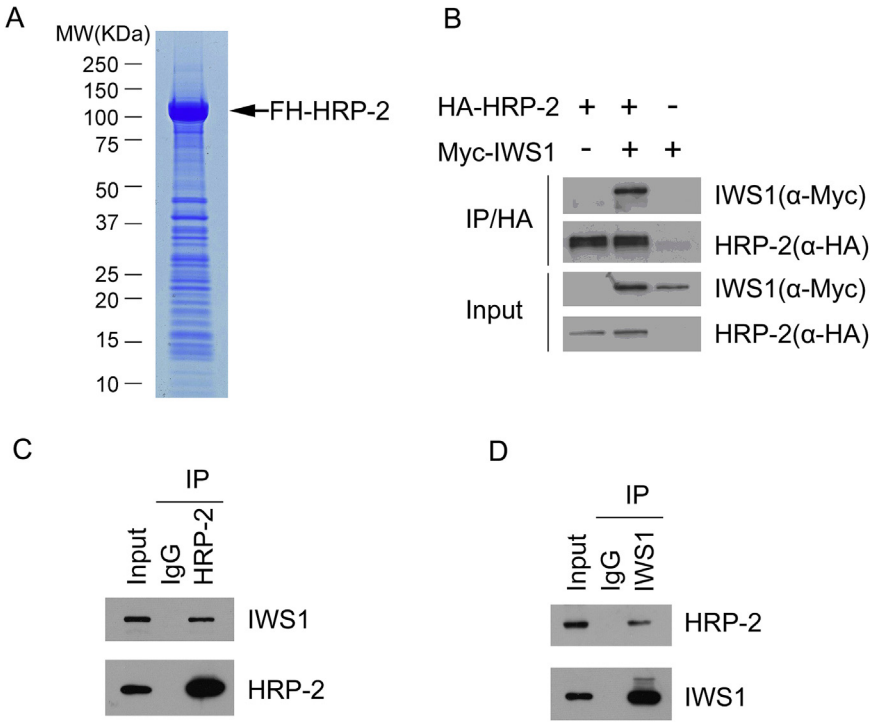

Fig. 3. HRP-2 forms a complex with IWS1. (A) Tandem affinity purification of ASPP1/2-containing protein complexes were conducted using 293T cells stably expressing FLAG-HA tagged HRP-2. Associated proteins were separated by SDS-PAGE and visualized by Coomassie Blue staining. The proteins and the number of peptides identified by mass spectrometry analysis are shown in the Table S1. (B) 293T cells were co-transfected with HA-HRP-2 and Myc-IWS1 constructs. After 24 h, cell lysates were prepared for co-immunoprecipitation (co-IP) with anti-HA antibody and Western Blotting (WB) analyses. (C, D) QGY-7703 cell lysates were prepared for co-IP with anti-HRP-2 antibody and WB analyses with indicated antibodies (C), cell lysates were also prepared for co-IP with anti-IWS1 antibody and WB analyses with indicated antibodies (D).

transferase SETD2 [9]. Gene-specific silencing of IWS1 by RNA interference reveals that IWS1 is essential for cell viability [10]. A recent study showed Akt3 and Akt1 phosphorylate IWS1 at the conserved Ser720/Thr721 site, promotes the recruitment of SETD2 to the RNA Pol II elongation complex. Importantly, Phospho-IWS1 controls FGFR2 alternative splicing and contributes to lung carcinogenesis [11].

As verification of this approach, we first examined whether HRP-2 can interact with IWS1 in cells. To do this, HA-HRP-2 and Myc-IWS1 constructs were co-expressed in 293T cells. Cell lysates were subsequently prepared for co-immunoprecipitation (Co-IP) with anti-HA antibody. As shown in Fig. 3B, Myc-IWS1 was immunoprecipitated by HA-HRP-2, suggesting an interaction between these two proteins. Next, we decided to extend our analysis by investigating whether endogenous HRP-2 and IWS1 can interact with each other. In this case, Immunoprecipitation using anti-HRP-2 antibody was performed using cell lysates prepared from QGY-7703 cells. As shown in Fig. 3C, endogenous IWS1 was efficiently immunoprecipitated by HRP-2. Similar results were also obtained in the reciprocal Co-IP experiment in which IWS1 was able to immunoprecipiate HRP-2 (Fig. 3D). These results suggesting these two proteins can also interact at their endogenous levels. Taken together, these results indicated that HRP-2 can form a complex with IWS1 in vivo.

3.4. HRP-2 and IWS1 promote cyclin D1 expression

We hypothesized HRP-2 may affect the expression levels of genes which are critical for HCC cells growth by modulating transcription elongation and mRNA processing. Since HRP-2 can markedly promote cell growth, we investigated whether HRP-2 can affect the expression of some cell cycle-related genes, including Cyclin B1, Cyclin D1, Cyclin E1, CDK1, CDK2, p21 and p27. HRP2 protein expression was depleted by two siRNAs in QGY-7703 cells, then the proteins levels of cell cycle-related genes were detected by Western blot. As shown in Fig. 4A, HRP-2 knockdown caused a markedly decrease in Cyclin D1 proteins level, but had no obvious effect on the protein levels of other cell cycle-related genes [12]. Importantly, knockdown of IWS1 in QGY-7703 cells caused similar effect on the protein levels of cell cycle-related genes like HRP-2 (Fig. 4A). Next, we performed qRT-PCR to measure the mRNA level of Cyclin D1 in HRP-2 or IWS1-depleted cells. As shown in Fig. 4B, the mRNA level of Cyclin D1 was markedly decreased in HRP-2 or IWS1-depleted cells. Moreover, when HRP-2 or IWS1 was ectopically overexpressed in SMMC-7721 cells, a moderate increase in the protein level of Cyclin D1 was observed (Fig. 4C). At last, we explored if IWS1 was required for HCC cells growth like HRP-2. As shown in Fig. 4D, knockdown of IWS1 by two independent siRNAs markedly suppressed QGY-7703 and SMMC-7721 cells growth when compared with control knockdown cells (Fig. 4B). This result was consistent with previous studies that IWS1 was required for cell viability in colon cancer cells (HCT116) [10].

Since Cyclin D1 plays a vital role in cancer cell cycle progression and is overexpressed in human HCCs [12], these results suggested that HRP-2 and IWS1 may cooperatively regulate the mRNA and protein levels of Cyclin D1, which partially explained the tumor-promoting function of HRP-2 and IWS1.

4. Discussion

In this study, we demonstrated HRP-2 was frequently overexpressed in human HCCs tissues at mRNA and protein levels, and

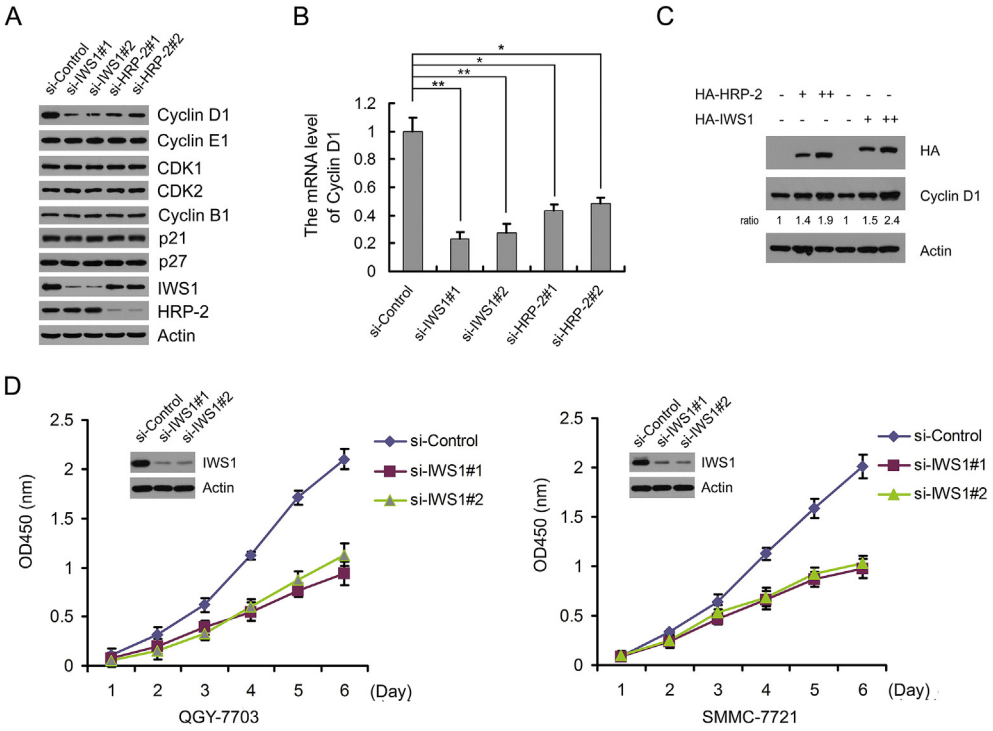

Fig. 4. HRP-2 and IWS1 controls cyclin D1 expression. (A) QGY-7703 cells were transfected with control or specific siRNAs towards HRP-2 or IWS1. After 48 h, cell lysates were prepared for WB analyses with indicated antibodies. (B) QGY-7703 cells were transfected with control or specific siRNAs towards HRP-2 or IWS1. After 48 h, the mRNA level of Cyclin D1 in HRP-2 or IWS1-depleted cells was measured by quantitative RT-PCR. The mean values (S.D.) of three independent experiments are shown. (C) SMMC-7721 cells were transfected with increasing doses of HA-HRP-2 or IWS1 constructs. After 24 h, cell lysates were prepared for WB analyses with indicated antibodies. (D) QGY-7703 (left panel) or SMMC-7721 (right panel) cells were transfected with control or two IWS1-specific siRNAs. After 24 h, the cell proliferation was measured at indicated days.

promote HCC cells growth in vitro and in vivo. These characteristics are very similar to those of HDGF and HRP-3. However, HRP-2 may exert its growth-promoting function through different molecular pathway. First, HRP-2 can't be secreted from cells as a mitogenic factor like HDGF or HRP-3 (data not shown). Second, HRP-2 encodes a larger protein than HDGF and HRP-3, and it is not surprising that HRP-2 has some unique binding partners, such as IWS1. HRP-2 may promote HCC progression by regulating transcription elongation and mRNA processing. The detailed functional relationship between HRP-2 an IWS1 remains be explored further. A previous study showed that PWWP proteins constitute a new family of methyl lysine histone binders. The PWWP domain of BRPF1 preferentially binds tri-methylated H3K36 (H3K36me3) [11]. Interestingly, IWS1 can recruits the SETD2 histone methyltransferase to the RNAPII elongation complex and is required for H3K36 trimethylation across the transcribed region of the c-Myc, HIV-1, and PABPC1 genes in vivo [9]. Taken together, we hypothesized that HRP-2 may form a complex with IWS1 and SETD2 to promote H3K36 trimethylation, which may be critical for transcription elongation and mRNA processing.

In addition to IWS1, our mass spectrometric analysis of HRP-2 protein complex detected many peptides corresponding to three subunits (α1, α2 and β) of casein kinase 2 (CK2) (Table. S1), raising the possibility that HRP-2 is a potential CK2 substrate. HRP-2 protein sequence contains many CK2 hierarchical consensus motifs (S/T-x-x-D/E/pS). Indeed, our preliminary results showed that HRP-2 protein can be easily phosphorylated by recombinant CK2 in an in vitro kinase assay (data not shown). Numerous studies have demonstrated CK2 plays critical role in gene transcription and RNA metabolism [13]. Thus, the functional impact of CK2-mediated HRP-2 phosphorylation needs to be addressed in further studies.

The findings from our study and others demonstrated three members of HRPs (HDGF, HRP-2 and HRP-3) were all overexpressed in HCC tissues, but may promote cancer cell progression through different molecular mechanism. Since these proteins showed high sequence similarity among HATH/PWWP domain, it may be promising to design small inhibitor targeting the HATH/PWWP domain to suppress HCC cell growth.

Acknowledgments

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Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.02.042.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.042.

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