Targeting Mybbp1a suppresses HCC progression via inhibiting IGF1/AKT pathway by CpG islands hypo-methylation dependent promotion of IGFBP5

Xiaoyu Weng, Jingbang Wu, Zhen Ly, Chuanhui Peng, Junru Chen, Cheng Zhang, Bin He, Rongliang Tong, Wendi Hu, Chaofeng Ding, Lining Cao, Diyu Chen, Jian Wu, Shusen Zheng

Article history:
Received 11 February 2019
Received in revised form 10 May 2019
Accepted 10 May 2019
Available online 17 May 2019

Keywords:
Myb-binding protein 1A (Mybbp1a)
IGF-binding proteins 5 (IGFBP5)
Hepatocellular carcinoma (HCC)
IGF1/AKT pathway
CpG islands methylation

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common solid tumors worldwide, and its incidence and mortality rate have persistently increased in recent years [1,2]. Surgical resection and liver transplantation (LT) are first-line treatments in early HCC. However, many patients are diagnosed with advanced HCC and are not eligible for surgery [3,4].

Furthermore, frequent metastasis and recurrence are the major reasons for the poor prognosis of HCC patients after surgical resection or LT [5]. Potential effective biomarkers for predicting the recurrence and prognosis of HCC are rare. Therefore, the identification of new molecular markers is needed.

MYB binding protein 1a (Mybbp1a) is a nuclear protein that can regulate rRNA metabolism, the stress response and carcinogenesis. However, the function of Mybbp1a in the progression of hepatocellular carcinoma (HCC) is unclear. We aimed to determine the role of Mybbp1a in HCC and the underlying mechanism.

Methods: We investigated the function of Mybbp1a in HCC cell models and the xenograft mouse model. The relationship between Mybbp1a and IGFBP5 was found through expression profile chip. The molecular mechanism of Mybbp1a regulating IGFBP5 was proved through CO-IP, CHIP, Bisulfite Sequencing and Pyrosequencing.

Findings: In this study, we observed that Mybbp1a was overexpressed in HCC tissues and associated with the poor prognosis of HCC patients. Suppression of Mybbp1a led to a reduction in the proliferation and migration ability of HCC cells through inhibiting the IGF1/AKT signaling pathway. Further study found that Mybbp1a could form a complex with DNMT1 and induce aberrant hyper-methylation of CpG islands of IGFBP5, which inhibits secretion of IGFBP5 and then activates IGF1/AKT signaling pathway.

Interpretation: These findings extend our understanding of the function of Mybbp1a in the progression of HCC. The newly identified Mybbp1a may provide a novel biomarker for developing potential therapeutic targets of HCC.

Fund: Science Technology Department of Zhejiang Province (No. 2015C03034), National Health and Family Planning Commission of China (No. 2016138643), Innovative Research Groups of National Natural Science Foundation of China (No. 81721091), Major program of National Natural Science Foundation of China (No. 91542205).

© 2019 Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

https://doi.org/10.1016/j.ebiom.2019.05.029
2352-3964/© 2019 Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
2.2. Cell culture

The HCC cell line SK-Hep-1 was purchased from the American Type Culture Collection (Manassas, VA). The liver cancer cell lines Huh7 and HCC-LM3 and the normal liver cell line QSG-7701 were purchased from the Chinese Academy of Science Cell Bank (Shanghai, China). All cell lines were proven by short tandem repeats (STR) and SNP fingerprinting before use. Cells were maintained in MEM or RPMI 1640 medium supplemented with 10% FBS at 37 °C in a humidified atmosphere of 5% CO2. The AKT inhibitor MK2206 was obtained from Selleck Chemicals (Houston, TX) and dissolved in ddH2O for the purpose of this research. IGFI and IGFBP5 were obtained from Peprotech (Rocky Hill, USA) and dissolved in ddH2O with 3% BSA for research.

2.3. Construction of stably transfected cell lines

The target sequences of Mybbp1a (5′-GCUGUGAAGUGUCUGAAAG AUGGCC-3′), and IGFBP5 (5′-CTCCCATTCTCCAATGACT-3′) were obtained from Shanghai Genechem Co. Ltd. and used to generate stable Mybbp1a or IGFBP5 knockdown cell lines according to the instructions of manufacturer. Cells were selected by puromycin (4 μg/ml) (Sangon, Shanghai, China) for 72 h, and the transfection efficiency was proved by quantifying EGFP fluorescence, RT-PCR and western blotting. Stably transfected Huh7 and HCC-LM3 cells were maintained in the Modified Eagle medium (MEM, Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS, Gibco) for further study.

2.4. Real-Time PCR and western blot

Real-Time PCR and western blot were conducted as previously described [19–21]. The primers were as follows: Mybbp1a, Forward 5′-GACATCTGACAGCAGATACAA-3′; Reverse 5′-CAGCTTCAGGACTTC ATCA-3′; IGFBP5, Forward 5′-TGACCGAAGAAGATCTCAAG-3′; Reverse 5′-CCTCAACGATCTCCATGTC-3′; and GAPDH, Forward 5′-GAAT GGGAAAGGTGAAGGTCG-3′; Reverse 5′-GGGTCATTGATGCGCAACA ATA-3′. The antibodies are shown in the Supplement Table 1.

2.5. Immunohistochemistry

Immunostaining (IHC) was performed on formalin-fixed, paraffin-embedded clinical tissue specimens. The expression levels of Mybbp1a in HCC tissues and neighbor tissues were detected as the previously described [19]. The IHC score was assessed by two independent investigators. The method for the IHC score was as follows: 0, negative; 1, <25% positive tumor cells; 2, 25–50% positive tumor cells; 3, 50–75% positive tumor cells; and 4, >75% positive tumor cells. The intensity of the dye color was graded as 0 (no color), 1 (light yellow), 2 (light brown), or 3 (brown). The staining index was calculated as follows: staining index = staining intensity × tumor cell staining grade. High Mybbp1a expression levels were defined as a staining index score ≥ 6, while low expression was defined as a staining index < 6.

2.6. Cell proliferation and migration experiments in vitro

HCC cell lines with Mybbp1a knockdown, Mybbp1a + IGFBP5 knockdown or no knockdown (negative control) were used for cell proliferation and migration assays as previously described [20,21].

2.7. Immunofluorescence staining

The HCC cell line Huh7-shMybbp1a, Huh7-NC, HCC-LM3-shMybbp1a and HCC-LM3-NC cells were fixed with 4% paraformaldehyde for 15 min and then permeabilized and simultaneously blocked with 0.1% Triton-X 100 and 1% BSA for 30 min. Cells were incubated sequentially with the Phalloidin-iFluor 647 conjugate (AAT Bioquest®, Inc., CA) for 1 h at
room temperature and then with DAPI for 5 min. Finally, the cells were examined with a laser confocal microscope (FV1000, Olympus).

2.8. ELISA assay

The Human IGFBP-5 ELISA Kit was purchased from Ray Biotech, Inc. (ELH-IGFBP5-1, P24593), and the experiment was conducted according to the guidelines.

2.9. Tumor xenograft and lung metastasis

All experimental protocols were approved by the Animal Experimental Ethics Committee of the First Affiliated Hospital of Zhejiang University School of Medicine. The methods were carried out in accordance with the approved guidelines. Cells (5 × 10^6) were resuspended in 100μl of PBS and injected subcutaneously into the lateral flanks of immunodeficient mice. Tumor volumes were measured every three days and calculated using the following equation: V (cm³) = width² (cm²) × length (cm)/2. The mice were sacrificed about five weeks after the injection. In addition, the nude mice were each injected via the tail vein with Mybbp1a-shRNA or Mybbp1a-NC (1 × 10^6 cells). Mice were sacrificed 40 days after the tail vein injection, and the lungs were embedded in paraffin and sliced for HE staining to identify metastasis.

2.10. Expression microarrays

Huh7 cells were transfected with shMybbp1a and negative control respectively. After RNA preparation using RNeasy Protect Midi Kit (Qiagen, Valencia, CA), hybridization was conducted using Affymetrix whole-genome microarrays (hgu133Plus2) according to the guidelines.

Fig. 1. Mybbp1a was overexpressed in HCC tissues and associated with poor prognosis of HCC patients. (a) Showed the expression of Mybbp1a in HCC tissues and adjacent non-tumor tissues (200×, scale bar = 0.1 mm; 400×, scale bar = 0.05 mm), and the results were quantified in the pie chart. (p < .0001, student’s t-test). (b) Showed the expression level of Mybbp1a in HCC cell lines and normal liver cells, (***p < .0005, **p < .005, student’s t-test). The overall and relapse-free survival of HCC patients between Mybbp1a high-expression group and Mybbp1a low-expression group were analyzed by Kaplan-meier analysis, (p = .014 and p = .010 respectively, Log Rank (Mantel-Cox)).
the manufacturer's instructions. Raw data were normalized using the RMA algorithm in R statistical computing environment (www.r-project.org) using the default parameters. The relative fold changes were calculated according to negative control.

### 2.11. Bisulfite sequencing and pyrosequencing

Genomic DNA of cells was extracted using the standard phenol–chloroform technique followed by proteinase K treatment to prevent protein contamination. Bisulfite conversion was performed using an EpiTect Bisulfite Kit (QIAGEN) according to the manufacturer's instructions. The primers for IGFBP5 amplification were as follows: forward 5'-ATGTGTTTTTATTTTTTTTGTTG-3'; reverse 5'-ACAAAATCTCCCAAACCTCTATTAG-3'.

For pyrosequencing, the amplified DNA products were detected by PyroMark Q96 ID (QIAGEN). The primers for IGFBP5 amplification were as follows: forward 5'-TTGGGTTAGAGTTGGTTAAGGA-3', reverse 5'-ACCCCAAATCCCAAACCTACTAC-3’. The primer for sequencing was as follow: 5'-TGAGAGATTAGGTGGTTAAGTAG-3'.

### 2.12. Co-immunoprecipitation

Co-immunoprecipitation was performed as previously described [22].

---

**Fig. 2.** Suppression of Mybbp1a inhibited proliferation of HCC cells via G1/S transition. (a) The transfection efficiency of shMybbp1a and negative control (NC) in Huh7 and HCC-LM3 cells was verified both at protein and mRNA levels, (**p < .005, student’s t-test**). (b) The Edu assay was used to detect proliferation rate of HCC cells with shMybbp1a or negative control (NC), (scale bar = 10um) (**p < .0005, **p < .005, student’s t-test**). (c) The tumorigenic ability of Huh7-shMybbp1a and Huh7-NC was compared both in vitro and in vivo, (**p < .005, ***p < .0005, student’s t-test**). (d) The cell cycle was detected by flow cytometry, and the results were analyzed by ModFIT software, (**p < .0005, **p < .005, student’s t-test**). (e) The expression of G1 phase associated proteins was detected by western blot, and β-actin was as the internal control.
2.13. Chromatin Immunoprecipitations-PCR

Chromatin Immunoprecipitations-PCR was conducted by Shanghai Kangchen Bio-tech, and the IGFBP5 primers was showed in the Supplementary Table 2.

2.14. Statistical analysis

Data are presented as the means ± standard deviation (SD). For comparisons, Student’s t-test and Fisher’s exact test were performed, as appropriate. Relapse-free survival and overall survival analysis were evaluated by the Kaplan-Meier method, and differences were assessed using the log-rank test. Cox multivariate regression analysis was used to identify independent prognostic factors. All analyses were performed with SPSS 18.0 software (SPSS Inc., Chicago, IL). Differences were considered significant at P < .05.

3. Results

3.1. Mybbp1a is overexpressed in HCC tissues and associated with a poor prognosis of HCC patients

To investigate the clinical significance of Mybbp1a expression, we measured its expression in 71 paired HCC tissues and adjacent non-tumor tissues. Immunohistochemical (IHC) assays showed that the average expression level of Mybbp1a was significantly higher in HCC tissues than that in adjacent non-tumor tissues (Fig. 1a). We also observed that the expression of Mybbp1a was higher in HCC cell lines (SK-Hep-1, Huh7, HCC-LM3) than that in normal liver cells (QSG-7701) (Fig. 1b). Kaplan-Meier analysis showed that higher expression of Mybbp1a was associated with shorter overall survival and tumor-free survival in HCC patients (P = .014 and P = .010, respectively) (Fig. 1c). This result was validated by liver cancer survival analysis in human protein atlas database (www.proteinatlas.org) (Supplement Fig. 3).
Fig. 1). Cox multivariate regression analysis indicated that high expression of Mybbp1a was an independent and significant factor for overall and tumor-free survival (Supplement Table 2). Taken together, these data indicated that Mybbp1a was overexpressed in HCC and that its overexpression predicted a poor prognosis in HCC patients and might contribute to the progression of HCC.

3.2. Suppression of Mybbp1a inhibits the proliferation of HCC cells via G1/S transition

According to the expression level of Mybbp1a in HCC cell lines, we stably transfected Mybbp1a-shRNA into the Huh7 and HCC-LM3 cell lines, and the transfection efficiency was proven at both the protein and mRNA levels (Fig. 2a). Loss of function experiments showed that suppression of Mybbp1a led to lower cell viability and a lower proliferation rate in both Huh7 and HCC-LM3 cells (Supplement Fig. 2, Fig. 2b). Consistently, tumor formation assays showed that suppression of Mybbp1a significantly inhibited the tumorigenesis of HCC cells both in vitro and in vivo (Fig. 2c–d, Supplement Fig. 3). Flow cytometry analysis revealed that knockdown of Mybbp1a induced G1 phase arrest (Fig. 2e, Supplement Fig. 4). We also detected several G1 phase-associated proteins and discovered that suppression of Mybbp1a induced lower expression of CDK2, cyclin A2, CDK4 and cyclin E1, but higher expression of p21 (Fig. 2f, Supplement Fig. 5). These results indicated that Mybbp1a played an important role in the proliferation and regulation of HCC cells.

3.3. Suppression of Mybbp1a inhibits the migration ability of HCC cells in vitro and in vivo

Next, we evaluated whether Mybbp1a played a role in the motility of HCC cells. The results of transwell and wound-healing assays showed that suppression of Mybbp1a led to a lower migration ability than that of the negative control (Fig. 3a-b, Supplement Fig. 6). Consistently, a pulmonary metastasis model showed that the Huh7-shNC group displayed more lung metastasis foci and larger metastatic tumors than those of the Huh7-shMybbp1a group (Fig. 3c). Then, we used phalloidin to observe the cell cytoskeleton in HCC cells and found that the numbers of microfilaments and microtubules were lower in the Huh7-
3.4. Mybbp1a promotes the proliferation and migration of HCC cells via PI3K/AKT pathway

To further explore the mechanism of Mybbp1a in regulating the proliferation and migration of HCC cells, we used Affymetrix whole-genome microarrays (hgu133Plus2) to investigate the differential genes and pathways between the Huh7-shMybbp1a and Huh7-NC groups. KEGG enrichment analysis showed that the PI3K/AKT signaling pathway was significantly changed among the classic biological regulatory pathways (Fig. 4a). Then, we detected the expression of pAKT473, pAKT308, PI3K-P110 and PI3K-P85 in Huh7 and HCC-LM3 cells and found that all of these molecules were significantly decreased under the condition of Mybbp1a suppression (Fig. 4b). To further demonstrate our findings, we used MK2206-2HCL (an inhibitor of AKT) to conduct a remedy experiment, and the inhibition efficiency was validated by western blot analysis (Fig. 4c). Then, we detected cell viability and migration ability in HCC-shMybbp1a and HCC-NC cells with or without 5
μmol/l MK2206-2HCL by the CCK-8 and transwell assays. The results showed that, except for the negative control (NC) group, the cell viability of the other groups was similar at 72 h (Fig. 4d). Consistently, this phenomenon also appeared in a cell migration experiment (Fig. 4e). In addition, we also demonstrated that inhibition of AKT activation induced a change in the expression of G1 phase-associated proteins and cytoskeletal synthesis reduction (Supplemental Figs. 7–8). Thus, our study proved that Mybbp1a regulated the proliferation and migration of HCC cells by promoting the activation of the PI3K/AKT signaling pathway.

3.5. IGFBP5 is negatively regulated by Mybbp1a in HCC cells

To further investigate the mechanism of Mybbp1a in the activation of the PI3K/AKT pathway, we analyzed the differential gene expression in our Affymetrix whole-genome microarrays. We found that IGFBP1, HAL, PPP2R2D, UCA1, WIF1, IGFBP5 and IGFBP2 had large fold changes under Mybbp1a suppression (Fig. 5a). A recent study proved that IGFBP5 was a potent inhibitor of both the signaling and functional outputs of IGF-1, which plays a vital role in the activation of the PI3K/AKT pathway [23]. Thus, we selected IGFBP5 for further study. First, we validated that IGFBP5 was significantly increased among the mRNA, intracellular protein and extracellular protein levels under Mybbp1a knockdown in Huh7 cells (Fig. 5b). Then, we analyzed patients with Mybbp1aHighIGFBP5Low, Mybbp1aLowIGFBP5High, Mybbp1aHighIGFBP5High or Mybbp1aLowIGFBP5Low. The results showed that the overall and tumor-free survival of patients with Mybbp1aHighIGFBP5Low was shorter than those with Mybbp1aLowIGFBP5High (Fig. 5c, Supplement Fig. 9). In addition, we constructed three stably transfected cell lines, Huh7-shMybbp1a, Huh7-shMybbp1a + shlIGFBP5 and a negative control, and the transfection efficiency was verified by western blotting (Fig. 5d). The CCK-8 assay showed that simultaneous knockdown of Mybbp1a and IGFBP5 reversed the decreased viability of cells induced by Mybbp1a knockdown alone (Fig. 5e). Consistently, the results of the transwell assay were similar to those of the cell viability experiment (Fig. 5f). Based on the above findings, we demonstrated that IGFBP5 was negatively regulated by Mybbp1a in HCC cells.

![Fig. 6. Mybbp1a promoted activation of IGF1/IGF1R/PI3K/AKT through inhibiting secretion of IGFBP5. (a) The expression level of pAKT473, AKT, pIGF1R and pIGF1R were detected in Huh7 cells treated with IGF1 (40 ng/ml), IGF1 + IGFBP5 (40 ng/ml) or the control. (b) Cell viability of Huh7 cells treated with IGF1 (40 ng/ml), IGF1 + IGFBP5 (40 ng/ml) or the control was detected, and the results of 72 h were used for statistical analysis, (**P<.005, one-way ANOVA). (c) Cell migration capacity of Huh7 cells treated with IGF1 (40 ng/ml), IGF1 + IGFBP5 (40 ng/ml) or the control was detected, (**P<.005, student’s t-test). (d) Expression level of pAKT473, AKT, pIGF1R and pIGF1R were detected after treatment with IGF1 0 ng/ml or 40 ng/ml. The results were quantified by Image J, (**P<.05, **P<.005 and ***P<.0005, student’s t-test).](image-url)
3.6. Mybbp1a promotes the activation of the IGF1/AKT signaling pathway by inhibiting the secretion of IGFBP5

The secretion of IGFBP5 inhibited the activation of IGF1R by competitive binding with IGF1 in the extracellular space [23]. Thus, we hypothesized that the inactivation of the PI3K-AKT pathway was mainly due to the inhibition of the IGF1 pathway in the case of Mybbp1a knockdown. We first validated that the phosphorylation of IGF1R and AKT by IGF1 (Peprotech, Product number 100-11) was inhibited after adding the recombinant protein IGFBP5 (Peprotech, Product number 100-05) (Fig. 6a). Then, the CCK-8 assay showed that the cell viability of that IGF1 group was higher than that of IGFBP5 and IGF1 + IGFBP5 groups (Fig. 6b). Similarly, this phenomenon appeared in the cell migration experiment (Fig. 6c). Moreover, in comparison with no IGF1 treatment, we validated that the expression of pIGF1R and pAKT473 both increased under the condition of 40 ng/ml IGF1 in Huh7 cells with shMybbp1a, shMybbp1a + shIGFBP5 or a negative control. However, the expression of pIGF1R and pAKT473 in cells with shMybbp1a + shIGFBP5 and negative controls was higher than that of those with shMybbp1a (Fig. 6d). Taken together, these findings showed that suppression of Mybbp1a led to the inactivation of the IGF1/AKT pathway by inducing the secretion of IGFBP5.

3.7. Mybbp1a inhibits the transcription of IGFBP5 by promoting CpG island methylation

As the suppression of Mybbp1a significantly increased the mRNA level of IGFBP5, we sought to determine how Mybbp1a affected the expression of IGFBP5, through transcriptional level or post-transcriptional level. First, we conducted microRNA sequencing to search for candidate microRNA which affected expression of IGFBP5 in post-transcriptional level. Considering that the mRNA level of IGFBP5 was upregulated under suppression of Mybbp1a, we selected miRNAs that were decreased in the Huh7-shMybbp1a group. Based on the above findings,

![Fig. 7. Mybbp1a inhibits transcription of IGFBP5 via promoting CpG islands methylation.](image-url)

(a) CpG island methylation level of IGFBP5 in HCC tissue and normal tissue was obtained from TCGA database, and relationship between CpG island methylation level and mRNA level of IGFBP5 was analyzed, \( p = 0.0041 \) (student’s \( t \)-test and \( p = 0.056 \), Pearson) (b) CpG sites of IGFBP5 was showed, TSS indicates transcription start site. And the percentages of DNA methylation in specific sites of the IGFBP5 CDS were analyzed by pyrosequencing, \( (*p < .05, \text{student’s } t\text{-test}) \) (c–d) The percentages of DNA methylation in specific sites of the IGFBPS CDS were analyzed by bisulfite sequencing PCR in HCC stable cell line with/without Mybbp1a knockdown, or HCC cell lines treated with 5Aza (0, 10, 20, 40 \( \mu \)mol/L) for 48 h. (e) The mRNA level of IGFBP5 was detected under different concentration of 5Aza (0, 10, 20, 40 \( \mu \)mol/L) after 48 h, \( (*p < .05, **p < .0005, \text{student’s } t\text{-test}) \). (f) Expression of DNMT1 under different concentration of 5Aza (0, 10, 20, 40 \( \mu \)mol/L) for 48 h was detected.
five miRNAs (miR-3187-3p, miR-210-5p, miR-671-5p, miR-1260b and miR-128-1-5p) were found (Supplement Fig. 10a). However, there was no significant change in the IGFBP5 mRNA level under treatment with miRNA mimics in Huh7 cells (Supplement Fig. 10b). Bioinformatics analysis (MethPrimer 2.0) showed that the CpG islands of IGFBP5 were not in the promoter site, while found one in the CDS (coding sequence) site (Supplement Fig. 11). Then we found that the methylation of CDS CpG island of IGFBP5 in HCC tissues was higher than that in normal tissues, and mRNA level of IGFBP5 was negative correlated with methylation level of CDS CpG island methylation. By analyzing the methylation level of 40 CpGs in the IGFBP5 CDS site through Bisulfite Sequencing PCR, we observed that the CpG methylation level of the negative control was higher than that of the shMybbp1a group, ranging from 26 CpG to 36 CpG. Then, we quantified the methylation percentage of these 11 CpGs (+259–+310) by pyrosequencing. The results showed that the methylation levels at position 1, 4, 5, 7, and 10 were significantly decreased under Mybbp1a suppression (Fig. 7b). As the results of Bisulfite Sequencing PCR showed that the methylation levels of position 7–11 were significantly different between the NC group and shMybbp1a group in HCC cell lines (Fig. 7c); thus, we selected these five CpG sites for further study. Then, we used Azacitidine (5Aza) to treat the HCC cells, and the results of Bisulfite Sequencing PCR showed that the methylation levels of position 7–11 were decreased (Fig. 7d). In addition, we found that the mRNA level of IGFBP5 was significantly increased after treatment with 5Aza (0, 10, 20, 40 μmol/l) (Fig. 7e). The inhibition efficiency of 5Aza was verified by detecting the expression of DNMT1 under different concentrations of 5Aza (Fig. 7f). Therefore, our study demonstrated that Mybbp1a inhibited the transcription of IGFBP5 by promoting the methylation of CpG islands in the CDS site.

3.8. Mybbp1a promotes the methylation of CpG islands of IGFBP5 through DNMT1

To demonstrate the mechanism of Mybbp1a’s regulation of the methylation of CpG islands of IGFBP5, we conducted Chip-PCR analysis to determine whether Mybbp1a and DNMT1 could combine with the CDS site of IGFBP5. We designed 6 primers according to the CDS site of IGFBP5, and the results showed that all of the primers had PCR products (Fig. 8a–b). In addition, the result of Co-IP showed that Mybbp1a could also bind to DNMT1 in HCC cell lines (Fig. 8c). Thus, our study proved that Mybbp1a was able to bind to the CDS site of IGFBP5 and then induce methylation of the CpG site by recruiting DNMT1.
4. Discussion

MYB binding protein 1a (Mybbp1a), a nucleolar transcriptional regulator, was first identified as a binding protein with the MYB proto-oncogene protein [6,7]. Further studies demonstrated that Mybbp1a played a vital role in response to P53-associated nucleolar stress, the synthesis of ribosomal DNA and amino acids, and the progression of the oxidative phosphorylation respiratory chain [9–16,24]. However, the mechanism of Mybbp1a in tumor initiation and progression was controversial. It was shown that Mybbp1a acted as a tumor suppressor in breast cancer by enhancing p53-associated anokisis and inhibiting the deacetylation activity of H3K18Ac [17,25]. However, a recent study demonstrated that Mybbp1a enhanced tumor cell proliferation in the early phase of HNSCC and inhibited tumor cell migration and invasion in advanced HNSCC [18]. In addition, we found that Mybbp1a was overexpressed in HCC tissues by the Human Protein Atlas database. Based on these, we propose that Mybbp1a may have a special function in the initiation and progression of HCC.

We first demonstrated that Mybbp1a was overexpressed in HCC tissues and that the high expression of Mybbp1a was associated with poor overall survival and tumor-free survival. Then, loss-of-function experiments showed that suppression of Mybbp1a inhibited the proliferation and migration of HCC cells through G1 phase arrest and suppression of Cdc42-mediated cytoskeleton synthesis. To further illustrate this phenomenon, we used transcriptome analysis in HCC cells and found that the expression of PI3K/akt pathway-related genes had significantly changed. As the activation of PI3K/akt promoted the progression of the cell cycle and cytoskeleton synthesis [26–30]. And our study validated that suppression of Mybbp1a decreased the expression of pAkt89, pAKT473, PI3K-P110 and PI3K-P85. In addition, the cell viability and migration capacities of HCC cells with Mybbp1a knockdown were similar to those of HCC cells under the condition of AKT inhibitors. Thus, we proved that Mybbp1a regulated the proliferation and migration of HCC cells via the PI3K-AKT pathway.

To further illustrate the mechanism of Mybbp1a on the activation of the PI3K-AKT pathway, we analyzed the differentially expressed genes on the transcriptome chip and found that the expression of IGFBP5 was significantly increased. IGFBP5, one of the IGF-binding proteins (IGFBP-1 to -6), is a secreted protein that inhibits the IGF1/IGF1R/PI3K/AKT axis through competitive combination with circulating IGF1 [31]. Recent studies reported that IGFBP5 was a tumor suppressor in many solid tumors, including breast cancer [32], head and neck cancer [33], osteosarcoma [34], uterine leiomyoma [35], melanoma [36], colorectal cancer [37], gastrointestinal stromal tumors [38] and papillary thyroid carcinoma [39]. Based on these data, we hypothesized that the deactivation of the PI3K/AKT pathway under the suppression of Mybbp1a might be due to the increased secretion of IGFBP5.

We first validated that the expression of IGFBP5 was increased both at the mRNA and protein levels under Mybbp1a knockdown. Then, we found that HCC patients with Mybbp1alaoloIGFBP5ho had shorter overall and tumor-free survival than those with Mybbp1alaoloIGFBP5ho. CCK-8 and transwell assays showed that the cell viability and migration capacity of HCC cells with Mybbp1a suppression were reversed when suppressing both Mybbp1a and IGFBP5. In addition, we demonstrated that IGFBP5 inhibited the IGF1-mediated activation of the PI3K-AKT pathway in HCC cells. The expression of piGF1R and pAKT473 in HCC cells with shMybbp1a was lower than that of with shMybbp1a + shIGFBP5 or negative controls under the condition of 40 ng/ml IGF1. In summary, we demonstrated that suppression of Mybbp1a inhibited the activation of the IGF1/IGF1R/PI3K/AKT axis through inducing the secretion of IGFBP5.

Thus, we sought to determine how the suppression of Mybbp1a could increase the mRNA level of IGFBP5 in HCC cells, through inhibiting mRNA degradation or promoting mRNA synthesis. First, we thought that suppression of Mybbp1a might induce changes in the expression of IGFBP5-related miRNAs. miRNAs are a class of highly conserved short RNAs of 21–23 nucleotides that induce the degradation of mRNAs by binding to the 3′ untranslated region (3′UTR) of their targeted mRNAs [40,41]. Then, we performed miRNA sequencing in HCC cells with shMybbp1a or the negative control and found five candidate miRNAs (miR-3187-3p, miR-210-5p, miR-671-5p, miR-1260b and miR-128-1-5p). However, the mimics of these miRNAs could not significantly decrease the mRNA level of IGFBP5 in HCC cells with shMybbp1a. Then, we tried to find a breakthrough from DNA methylation modification, which is the major epigenetic modification that involves alterations of the chromatin structure. Increasing evidence demonstrated that aberrant methylation of CpG islands in the tumor suppressor gene led to transcriptional silencing in cancer [42,43]. Based on these data, we analyzed the sequence of IGFBP5, including the promoter region, CDS site, 5′ UTR Exons, 3′ UTR Exons and Introns, and found that there were CpG islands in the CDS site of IGFBP5. Then, the results of Bisulfite Sequencing and Pyrosequencing showed that the methylation levels of specific sites significantly decreased under Mybbp1a suppression. In addition, we found that Mybbp1a was able to specifically bind to DNMT1 and the CDS site of IGFBP5. Thus, we demonstrated that Mybbp1a inhibited the secretion of IGFBP5 by increasing the methylation level of CpG islands of the CDS site by recruiting DNMT1.

In conclusion, our study found that Mybbp1a suppressed the transcription of IGFBP5 through the DNMT1-mediated hyper-methylation level of CpG islands in the IGFBP5 CDS site. This biological process led to a loss of the secretion of IGFBP5, which improved the combinative efficiency between IGF1 and IGF1R. Then, phosphorylation of IGF1R induced the activation of the PI3K-AKT pathway, which increased the proliferation rate and migration capacity of HCC cells (fig. S8d). Though the most of the published studies reported the negative role of Mybbp1a in various cancer types, however, high heterogeneity of tumors from different tissue sources indicated that Mybbp1a may act as different roles during progression of different solid tumors. Therefore, our findings raised an intriguing hypothesis that Mybbp1a might serve as a “switch” to regulate the secretion of IGFBP5 to cater to the needs of HCC progression.

However, our study only demonstrated Mybbp1a could affect the methylation of CpG island in CDS site of IGFBP5, further studies were needed to prove whether Mybbp1a could regulate other CpG islands methylation of IGFBP5 or regulate the transcription of IGFBP5 by other pathways?

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.05.029.

Author contributions

Xiaoyu Weng, Zhen Lv, Jian Wu and Shusen Zheng designed the study. Xiaoyu Weng, Jingbang Wu, Junru Chen, Bin He, Cheng Zhang, Rongliang Tong, Wendi Hu and Linqing Cao, Chaofeng Ding and Diyu Chen performed the experiments. Xiaoyu Weng, Zhen Lv and Jian Wu drafted the manuscript. Xiaoyu Weng, Zhen Lv and Chuanhui Peng analyzed the results and made pictures.

Acknowledgement

This study was supported by grant from Science Technology Department of Zhejiang Province (No. 2015C03034), National Health and Family Planning Commission of China (No. 2016138643), Innovative Research Groups of National Natural Science Foundation of China (No. 817212091), Major program of National Natural Science Foundation of China (No. 91542205). These funding sources had no role in the study design, data collection, data analysis, interpretation, or writing of this manuscript.

Consent for publications

I have obtained consent to publish from the participant to report individual patient data.
Availability of data and materials

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

Ethics approval and consent to participate

This study was approved by the ethics committee of the First Affiliated Hospital of Zhejiang University, and informed consent was obtained from all patients. All experimental protocols were approved by the Animal Experimental Ethics Committee of the First Affiliated Hospital of Zhejiang University School of Medicine.

Conflicts of interest statement

The authors do not have a commercial or other association with pharmaceutical companies or other parties that might pose a conflict of interest.

References

[1] Finn RS. Current and future treatment strategies for patients with advanced hepatocellular carcinoma: role of mTOR inhibition. Liver Cancer 2012;1(3–4):247–56.
[2] Cheng J, Xie H-Y, Xu X, Wu J, Wei X, Su R, et al. NDRG1 as a biomarker for metastasis, recurrence and poor prognosis in hepatocellular carcinoma. Cancer Lett 2011;310(1):35–45.
[3] Abou-Alfa GK. Sorafenib use in hepatocellular carcinoma: more questions than answers. Hepatology 2014;60(1):8–15.
[4] Desai JR, Ochoa S, Prins PA, He AR. Systemic therapy for advanced hepatocellular carcinoma: an update. J Gastroint Oncol 2017;8(2):243–55.
[5] Chow AK, Ng L, Lam CS, Wong SK, Wain TM, Cheng NS, et al. The enhanced metastatic potential of hepatocellular carcinoma (HCC) cells with sorafenib resistance. PloS one 2013;8(11):e78075.
[6] Tavner FJ, Simpson R, Tashiro S, Favier D, Jenkins NA, Gilbert DJ, et al. Molecular clonality of hepatocellular carcinoma (HCC) cells with sorafenib resistance. Exp Cell Res 2003;289(1):108–23.
[7] Keough RA, Macmillan EM, Lutwyche JK, Gardner JM, Tavner FJ, Jans DA, et al. Myb-binding protein 1a is a nucleocytoplasmic shuttling protein that utilizes CRM1-dependent and independent nuclear export pathways. Exp Cell Res 2003;289(1):108–23.
[8] Keough R, Woolatt E, Crawford J, Sutherland GR, Plummer S, Casey G, et al. Myc-dependent gene expression. J Biol Chem 2002;277(25):22515–9.