

HBV Infection Promotes The Occurrence and Development of Hepatocellular Carcinoma Through Impairing The Inhibitory Effect of PPP2R5A on MAPK/AKT/WNT Signaling Pathway

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Research

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Abstract

Background: Reversible phosphorylation and dephosphorylation play important roles in cell function and cell signal transduction. PPP2R5A is responsible for specifically regulating the catalytic function, substrate specificity and intracellular localization of the tumor suppressor phosphatase PP2A. Therefore, the abnormal expression and function of PPP2R5A may be related to the occurrence and development of tumors.

Results: This study showed that PPP2R5A inhibited the proliferation and metastasis of hepatocellular carcinoma (HCC) cells, and acted as a tumor suppressor in HCC cells, but it had no significant effect on cell cycle. Further research found that PPP2R5A exerted tumor suppressor efficacy by inhibiting the MAPK/AKT/WNT signaling pathway. Combined with analysis of clinical tissue samples and TCGA database, it was found that the expression of PPP2R5A in tumor tissues of Chinese HCC patients was down-regulated and significantly correlated with the progression-free survival (PFS) of HCC patients. On the contrary, PPP2R5A showed an up-regulation trend in HCC cases in TCGA database although its effect on PFS was the same with that in Chinese HCC patients. HBV infection is the main pathogenic factor of HCC in China. It was found that hepatitis B virus (HBV) infection reduced the content of PPP2R5A in cells.

Conclusions: It was concluded that HBV inhibited the initiation of the protective mechanism mediated by PPP2R5A, making the occurrence and progress of HCC more "unimpeded". This conclusion will further reveal the role of PPP2R5A in HBV-induced and HBV-unrelated HCC, therefore, providing clues for the prevention and treatment of the two types of HCC, respectively.

Background

China has a high incidence of hepatocellular carcinoma (HCC), while the vast majority of HCC are associated with HBV infection [1–3]. In recent years, the anti-HCC effects of molecular targeted drugs have attracted more and more attention and become one of the hot spots in anti-tumor research.

Reversible phosphorylation and dephosphorylation play important roles in the maintenance of cell homeostasis and the regulation of cell functions. About 50% of the serine/threonine dephosphorylation activity within cells is regulated by the phosphatase protein phosphatase 2A (PP2A). PP2A is a most abundant phosphatase in cells, and its expression accounts for 0.2-1% of the total cell protein [4–7]. Therefore, the expression level, substrate specificity, intracellular localization and enzyme activity of PP2A are closely related to cellular physiological state and pathological state. It is generally believed that PP2A is a tumor suppressor, which can negatively regulate cell division, inhibit protein synthesis and promote cell apoptosis, and is a potential target for tumor targeted therapy [8, 9].

Protein phosphatase 2 regulatory subunit B' alpha (PPP2R5A) is one of the regulatory subunits of PP2A, which specifically regulates the phosphatase activity, substrate specificity and intracellular localization of PP2A. It has been reported that PPP2R5A is involved in the negative regulation of tumor-related proteins such as Bcl2 [10], MAPK [11], AKT [12], c-Myc [13] and β -Catenin [14], and abnormalities of PPP2R5A are

often accompanied by occurrence of various diseases such as adenocarcinoma and epithelial cancer [15–18]. It has been reported that lentiviral infection will increase the degradation of PPP2R5A to promote virus replication [19]. The analysis of HCC samples in the TCGA database found that PPP2R5A is upregulated in HCC patients, while the expression of PPP2R5A is significantly related to the prognosis of HCC. The HCC patients with high expression of PPP2R5A have a longer progression-free survival (PFS). The above clues suggested that PPP2R5A may exist as a tumor suppressor in the development of HCC. This study will explore the role and molecular mechanism of PPP2R5A in HCC.

Methods

Cell lines and cell culture

Human immortalized hepatocyte lines L02, WRL-68 and Chang liver cell lines, human HCC cell lines QGY-7701, Huh-7, SMMC-7721, Hep3B, HepG2, HepG2.2.15, PLC/PRF/5 and HCC-LM3 are provided by the Chinese Academy of Sciences Stem Cell Bank (Shanghai, China). Cells were cultured in DMEM (Gibco, USA) containing 10% FBS at 37 °C and 5% CO₂.

HCC tissue microarray and IHC evaluation

Cancer and adjacent tissue samples from 110 cases of HCC patients were collected and made into HCC tissue microarray. The study has been approved by the Committee on Ethics of Medicine, Navy Military Medical University (Shanghai, China), and the patients have signed informed consents. The expression of PPP2R5A in HCC and adjacent tissues was detected by IHC. The AlgorithmS program of ImageScope software (Aperio) was used to calculate the "positive pixel" of the samples in tissue microarray. The calculation method of immunohistochemical score for each sample is $\text{Log}_{10}[255/I_{\text{avg}}]$, where I_{avg} represents the average intensity. The median of the cancer tissue IHC score was selected to distinguish between the high and low expression of PPP2R5A. Combined with the pathological and follow-up data, the correlations between PPP2R5A expression level and clinical indicators and survival period were analyzed.

Western blot

Western blot was performed according to the conventional experimental methods. The antibodies used and the dilution ratio are shown in supplementary table 1 (Table S1).

Plasmid construction and transfection

The PPP2R5A knockdown vector pLKO.1-PPP2R5A shRNA (shRNA forward: 5'-CCGGCACTGAATGAACTGGTTGAGTCTCGAGACTCAACCAGTTCATTCAGTGTTTTTTG-3'; shRNA reverse: 5'-AATTCAAAAACACTGAATGAACTGGTTG AGTCTCGAGACTCAACCAGTTCATTCAGTG-3') and the expression vector pLV-EF1 α -PPP2R5A-EGFP were constructed. Lipo3000 liposome transfection reagent was used to transfect vectors into HCC cells. The total cell proteins were collected at 48 h after transfection for Western blot.

CCK8 assay

The logarithmic growth phase cells were seeded in 96-well plates, the final concentration was 5,000 cells /100 μ L (3 replicate wells per group). After being cultured in an incubator for 48 h, cells of each well were added with 10 μ L of CCK8 reagent and incubated at 37 °C for 2 h in dark, then placed in an enzyme-linked immunoassay instrument to detect OD₄₅₀. The cell proliferation rate was calculated.

Colony formation assay

The logarithmic growth phase cells were seeded in 6-well plates, the final cell density was 1,000 cells /well, and placed in an incubator for cultivation. After 14 days, cells were fixed with 4% formaldehyde, then stained with crystal violet for 15–20 min, washed with PBS for 3–4 times, and then photographed and statistically analyzed.

Cell Cycle

The cells in the logarithmic growth phase were collected and fixed with pre-cooled 75% alcohol, and placed at 4 °C overnight. The cells were centrifuged to remove alcohol, washed twice with PBS, added with 200 μ L PI (BD Company, USA) staining solution to resuspend, then placed in dark to react for 30 minutes, and tested on the machine.

Transwell assay

The transwell chambers were coated with matrigel, and 5×10^4 cells were seeded into 200 μ L serum-free DMEM in the upper chamber. 500 μ L of media containing 20% FBS were added to the lower chamber of transwell. Then the plate was placed in incubator for static culture. After 48 h, the upper chamber cells were wiped off, the migrated and invasive cells were stained with crystal violet for 15 minutes, and photographed and recorded under a microscope.

Statistical analysis

The data were analyzed by SPSS 17.0 software package and expressed as mean \pm standard deviation (Mean \pm SD). Analysis of variance (ANOVA) was used to analyze the data. $p < 0.05$ indicates statistical significance. The statistical graphs were made with Graphpad Prism 5 software.

Results

Low expression of PPP2R5A in HCC tissue is significantly related to poor prognosis of patients

In order to explore the expression level of PPP2R5A in Chinese HCC patients, immunohistochemistry (IHC) detection was performed on tissue microarrays containing cancer and adjacent tissues from 110 HCC patients. The results showed that the expression of PPP2R5A in 77 cases (70%) of cancer tissues was lower than that in the adjacent tissues, and the expression of PPP2R5A in 25 cases (22.7%) did not

change significantly. The expression of PPP2R5A in 8 cases (7.3%) of cancer tissue was higher than that in adjacent tissues (Fig. 1a, b). The expression of PPP2R5A in each sample of tissue microarray was scored by ImageScope (Aperio) software. The results of statistical analysis showed that the expression score of PPP2R5A in HCC tissues was significantly lower than that in adjacent tissues, and the difference was statistically significant ($p < 0.001$) (Fig. 1c). Western Blot was used to detect the content of PPP2R5A in the total proteins of 12 pairs of HCC and adjacent tissues. It was found that the content of PPP2R5A protein in HCC tissues was lower than that in adjacent tissues (Fig. 1d). The above results indicated that the expression of PPP2R5A in HCC tissues of Chinese patients is down-regulated. Among the 366 HCC samples in the TCGA database, about 24% (87/366) of the tumor tissues showed high expression of PPP2R5A (Fig. S1) (<http://www.cbioportal.org/>), and others showed the same expression levels of PPP2R5A between HCC tissues and adjacent tissues.

After scoring HCC tissue microarray, the median score of cancer tissue (0.24) was selected as the criterion for distinguishing the high and low expression levels of PPP2R5A. Combined with pathological and follow-up data, the correlation between the expression level of PPP2R5A and the survival time of HCC patients was analyzed. The higher expression level of PPP2R5A corresponds to longer progression-free survival (PFS) and overall survival (OS) ($p < 0.05$) (Fig. 1e). Analysis of samples from the TCGA database also showed that the high expression of PPP2R5A corresponds to a long PFS (Fig. S1).

In summary, PPP2R5A is generally down regulated in cancer tissues of Chinese HCC patients, and it is significantly positively correlated with the PFS of HCC patients.

PPP2R5A inhibits the proliferation of HCC cells

PPP2R5A was knocked down or overexpressed in HCC QGY-7701 and Hep3B cells. Western blot results showed that knockdown or overexpression of PPP2R5A was effective in both cell lines (Fig. 2a). The results of colony formation assay showed that the number of colonies of HCC cells overexpressing PPP2R5A was significantly less than that of the control group, while the number of colonies of HCC cells that were knocked down in PPP2R5A expression was significantly more than that of the control group (Fig. 2b). CCK8 assay confirmed that the overexpression of PPP2R5A caused a decrease in the proliferation of QGY-7701 and Hep3B cells, while knockdown of PPP2R5A caused an increase in the proliferation of the two HCC cell sublines (Fig. 2c).

Western Blot results showed that the overexpression of PPP2R5A resulted in the down-regulation of cell proliferation related indicators such as Bcl-2 and c-Myc. The knockdown of PPP2R5A yielded the opposite results (Fig. 2d, e). The changes of protein molecules were consistent with the inhibition of cell proliferation by PPP2R5A.

PPP2R5A inhibits the metastasis of HCC cells but has no effect on cell cycle

The effects of PPP2R5A on cell cycle were detected by flow cytometry. The results showed that PPP2R5A had no significant effect on cell cycle (Fig. 3a). The results of wound healing and transwell assays

showed that the high expression of PPP2R5A inhibited the migration and invasion of HCC QGY-7701 and Hep3B cells, while the knockdown of PPP2R5A significantly promoted HCC cell metastasis (Fig. 3b, c).

HBV infection leads to the down-regulation of PPP2R5A expression

From the above results, it can be known that the expression of PPP2R5A in the cancer tissues from 70% of Chinese HCC patients is down-regulated and shows a certain tumor suppressor effect. Among the 366 HCC samples from TCGA database, about 24% of cancer tissues showed high expression of PPP2R5A (Fig. S1). Considering that there are great differences in the regional pathogenic factors of HCC, the majority of HCC patients from China are HBV positive, it is speculated that the down-regulation of PPP2R5A expression may be related to HBV infection.

In HCC and normal liver tissues, the expression of PPP2R5A in HBV-positive tissues is lower than that in HBV-negative tissues (Fig. 4a). The results of HCC tissue microarray analysis showed that the expression of PPP2R5A was not significantly correlated with gender, age, AFP value and TNM staging of HCC patients, but was significantly correlated with the expression of HBsAg and pathological grade ($p < 0.05$) (Table 1). Western Blot found that the content of PPP2R5A in HepG2.2.15 (HBV+) cells was lower than its parental HepG2 (HBV-) cells (Fig. 4b), suggesting that the expression of PPP2R5A might be inhibited by HBV infection. Western Blot results showed that PPP2R5A was generally low expressed in HCC QGY-7701, PLC/PRF/5, and Hep3B cell lines that were integrated by HBV in cell genome (Fig. 4c).

Table 1
Correlation analysis of PPP2R5A expression level and clinical data

Variables	Low (n = 55)	High (n = 55)	p-value
Gender, x (%)			0.567
Female	8(14.5)	6(10.9)	
Male	47(85.5)	49(89.1)	
Age, x (%)			0.34
≥ 50	30(54.5)	25(45.5)	
<50	25(45.5)	30(54.5)	
HBsAg, x (%)			0.039
Negative	3(5.5)	10(18.2)	
Positive	52(94.5)	45(81.8)	
AFP, x (%)			0.381
Negative, <20 µg/L	12(21.8)	16(29.1)	
Positive, ≥20 µg/L	43(78.2)	39(70.9)	
TNM stage, x (%)			0.175
I and II	29(56.4)	36(61.8)	
III and IV	26(43.6)	19(38.2)	
Pathological grade, x (%)			0.045
I and II	14(25.5)	24(43.6)	
III and IV	41(74.5)	31(56.4)	

It is concluded from the above results that HBV infection down-regulates the content of PPP2R5A in hepatocytes. However, the specific mechanism through which HBV regulates the content of PPP2R5A remains unclear and needs to be further studied.

PPP2R5A exerts tumor suppressor efficacy by inhibiting the MAPK/AKT/WNT signaling pathway

The MAPK and AKT signaling pathway play an important role in the process of tumor invasion and metastasis [20, 21]. The activation of WNT signaling pathway is also more common in cancer development [22, 23]. PPP2R5A is a regulatory subunit of intracellular phosphatase PP2A, responsible for the specific regulation of the enzymatic function, substrate specificity and intracellular localization of the

tumor suppressor phosphatase PP2A, and likely to participate in the regulation of the above-mentioned signaling pathways.

Western Blot was used to detect the phosphorylation levels of Raf, ERK and AKT in HCC cells after knocking down or overexpressing *PPP2R5A*. The results showed that the overexpression of *PPP2R5A* significantly reduced the phosphorylation levels of Raf, ERK and AKT, and down-regulated the expression of β -Catenin in cells. While the knockdown of *PPP2R5A* obtained the opposite results. The p-RAF, p-ERK, and p-AKT are all upstream factors that regulate β -Catenin. It is speculated that *PPP2R5A* inhibits the activation of the WNT pathway by inhibiting the MAPK and AKT signaling pathways, and promotes the degradation of β -Catenin, thereby exerting a tumor suppressor efficacy (Fig. 5a, b).

Discussion

PPP2R5A has been reported to be involved in the regulation of signaling pathways of tumorigenesis [24]. Studies have shown that PP2A negatively regulated the cell anti-apoptotic factor Bcl2, *PPP2R5A* assisted PP2A in its functions and promoted the occurrence of apoptosis [11, 25]. Yamamoto et al. reported that *PPP2R5A* helped PP2A locate the important proto-oncoprotein β -Catenin in the WNT pathway by binding to the scaffold protein Axin, and promoted β -Catenin degradation [26]. Rodriguez et al. found that the lack of function of the *PPP2R5A* protein family led to the hyperfunction of the protein kinase B (AKT) family and promoted the activation of the WNT pathway and the occurrence of tumors [12, 26]. In addition, studies have shown that after inhibited the expression of *PPP2R5A* by microRNA-218, the WNT pathway would be significantly activated [27]. *PPP2R5A* can also assist PP2A to localize to c-Myc protein and promote the dephosphorylation and degradation of c-Myc protein [24]. These oncoproteins play an important role in the unlimited proliferation, infiltration and metastasis of tumor cells, and cell death resistance.

It is worth noting that some studies have found that viral infection could affect the expression of *PPP2R5A*. After treated cells with ceramide or double-stranded RNA, eukaryotic translation initiation factor 2 alpha kinase 2 (PKR) itself activated and phosphorylated the 28th serine of *PPP2R5A*, which enhanced the inhibitory effect of PP2A on protein synthesis and induction of apoptosis, thereby inhibiting viral infection [28, 29]. However, hepatitis C virus (HCV) mRNA can promote Staufen1 to competitively inhibit the binding of viral mRNA and PKR, and block the autophosphorylation activation of PKR [30], which can lead to the decline of *PPP2R5A* expression and function. The Vif protein produced by lentiviral infection can recruit Cullin-5 (CUL5) E3 ubiquitin ligase to induce the ubiquitination and degradation of *PPP2R5A* [19, 31]. These examples demonstrated the antagonism of *PPP2R5A* and viral infection in the co-evolution of virus-host. However, the effect of HBV infection on *PPP2R5A* has not been reported yet.

Based on the above results, the molecular mechanism of *PPP2R5A* in the occurrence and development of HCC is speculated as follows (Fig. 6): *PPP2R5A* exerts a tumor suppressor efficacy in hepatocytes. Under HBV-uninfected circumstances, when hepatocytes become cancerous, as one of the protective mechanisms, cells will up-regulate the expression of *PPP2R5A*. The high expression of *PPP2R5A* can

inhibit the proliferation and metastasis of HCC cells. Therefore, the results of survival analysis showed that the higher the expression of PPP2R5A, the higher the PFS of HCC patients. But HBV is precisely the "nemesis" of PPP2R5A. When HCC patients are infected with HBV, HBV can down-regulate the content of PPP2R5A in cells, leading to a weakening of cellular self-protection mechanism. At this time, the expression of p-RAF, p-ERK, and p-AKT is up-regulated, and the activation of MAPK and AKT signaling pathways leads to an increase of β -Catenin, the key molecule of WNT signaling pathway, and an activation of WNT signaling pathway. The continuous activation of MAPK/AKT/WNT signaling pathways can promote cell proliferation and metastasis, thereby promoting the occurrence and development of HCC.

Combined with the fact that 85%-90% of HCC in China is closely related to HBV infection, it may be that HBV infection weakens the self-protection function of hepatocytes and increases the possibility of cell canceration.

Conclusions

PPP2R5A acted as a tumor suppressor in HCC cells. HBV inhibited the initiation of the protective mechanism mediated by PPP2R5A, making the occurrence and progress of HCC more "unimpeded". This conclusion will further reveal the molecular mechanism of HBV-induced HCC and provide clues for the prevention and treatment of HCC induced by HBV infection.

Abbreviations

HCC: hepatocellular carcinoma; PFS: progression-free survival; HBV: hepatitis B virus; PP2A: phosphatase protein phosphatase 2A; PPP2R5A: protein phosphatase 2 regulatory subunit B' alpha; IHC: immunohistochemistry; OS: overall survival; PKR: eukaryotic translation initiation factor 2 alpha kinase 2; HCV: hepatitis C virus; CUL5: Cullin-5.

Declarations

Ethics approval and consent to participate

The study has been approved by the Committee on Ethics of Medicine, Navy Military Medical University (Shanghai, China), and the patients have signed informed consents.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files. Further details are available from the corresponding author upon

request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

XJ-L: responsible for the study design; acquisition of data; analysis and interpretation of data; the manuscript writing. ZM-M: responsible for study design; performing the experiments; statistical analysis. L-C and HH-Q were responsible for technical and material support. CY-L: drafting of the manuscript; critical revision of the manuscript for important intellectual content. CQ-S: drafting of the manuscript; critical revision of the manuscript for important intellectual content; study supervision.

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Figures

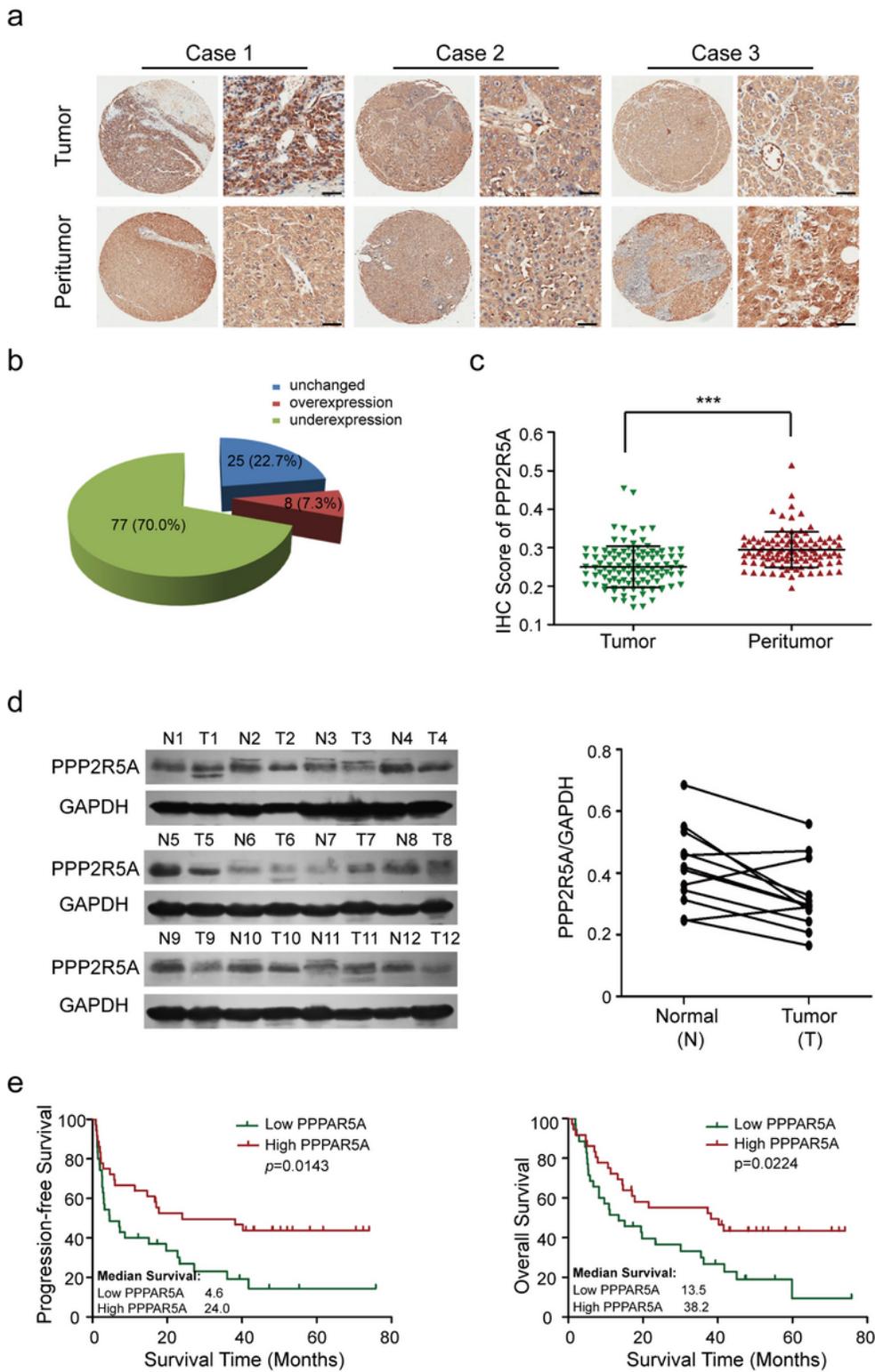


Figure 1

The low expression of PPP2R5A is significantly related to the poor prognosis of HCC. a. IHC results showed that the higher, lower and comparable expression of PPP2R5A were found in HCC tissues compared with peritumor tissues in the tissue microarray. Case 1: tumor>peritumor; Case 2: tumor≈peritumor; Case 3: tumor<peritumor. Bar=50 μm; b. Statistical analysis of the expression of PPP2R5A in HCC tissue samples relative to the adjacent tissues; c. IHC score results showed that the

expression of PPP2R5A in HCC tissues is lower than that of adjacent tissues (***, $p < 0.001$); d. Western blot of PPP2R5A in the total proteins of 12 pairs of HCC and adjacent tissues and semi-quantitative statistical analysis; e. Survival analysis showed that low expression of PPP2R5A was related to the poor prognosis of HCC patients.

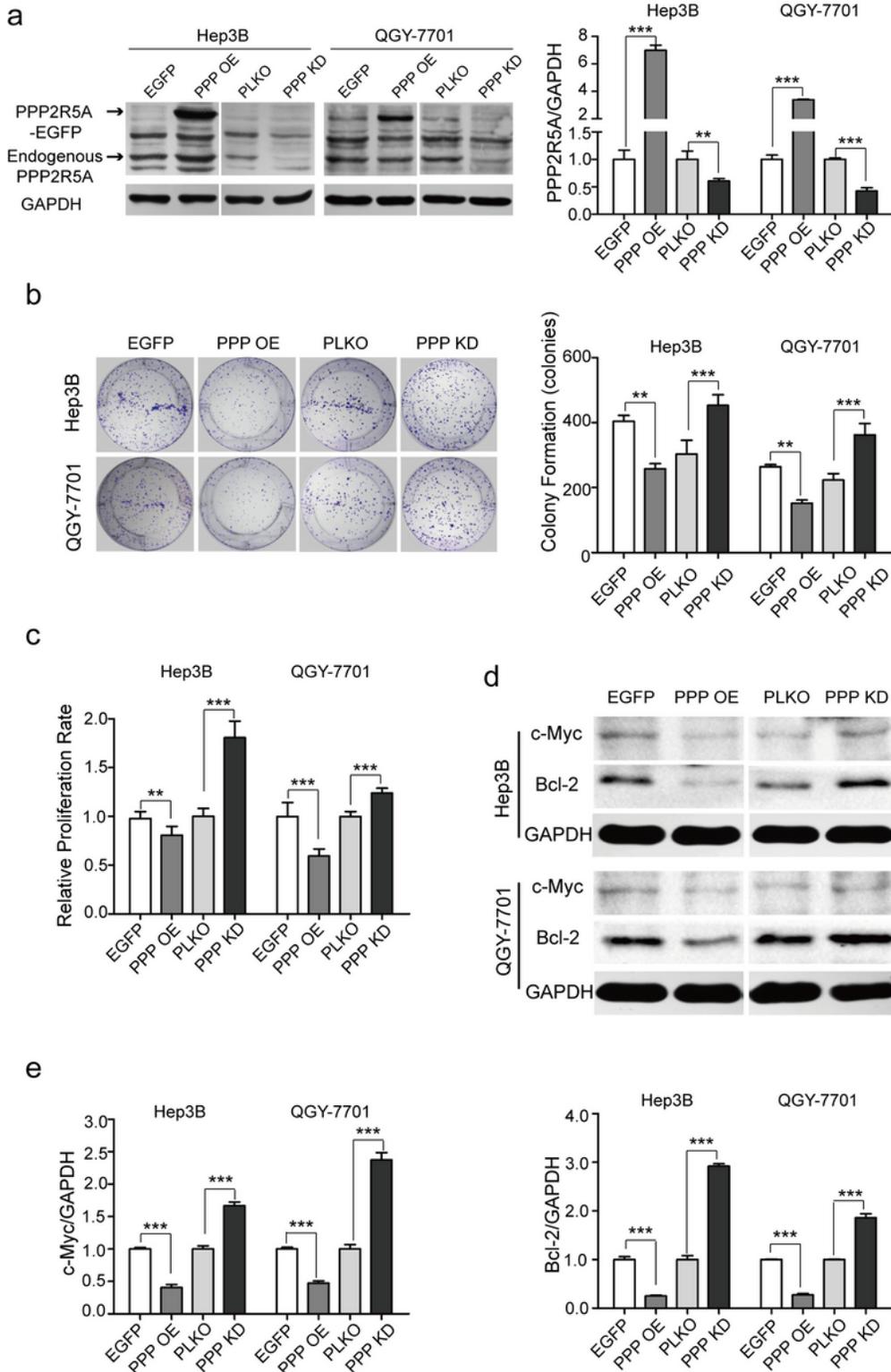


Figure 2

PPP2R5A inhibits the proliferation of HCC cells. a. Western Blot to verify the overexpression and knockdown of PPP2R5A in Hep3B and QGY-7701 cells; b. Colony formation assay to verify the effect of PPP2R5A on cell colony forming ability; c. CCK8 assay to verify the effect of PPP2R5A on cell proliferation; d and e. Western Blot to detect the cell proliferation related indicators such as Bcl-2 and c-Myc and the semi-quantitative statistical analysis. (**, $p < 0.01$; ***, $p < 0.001$)

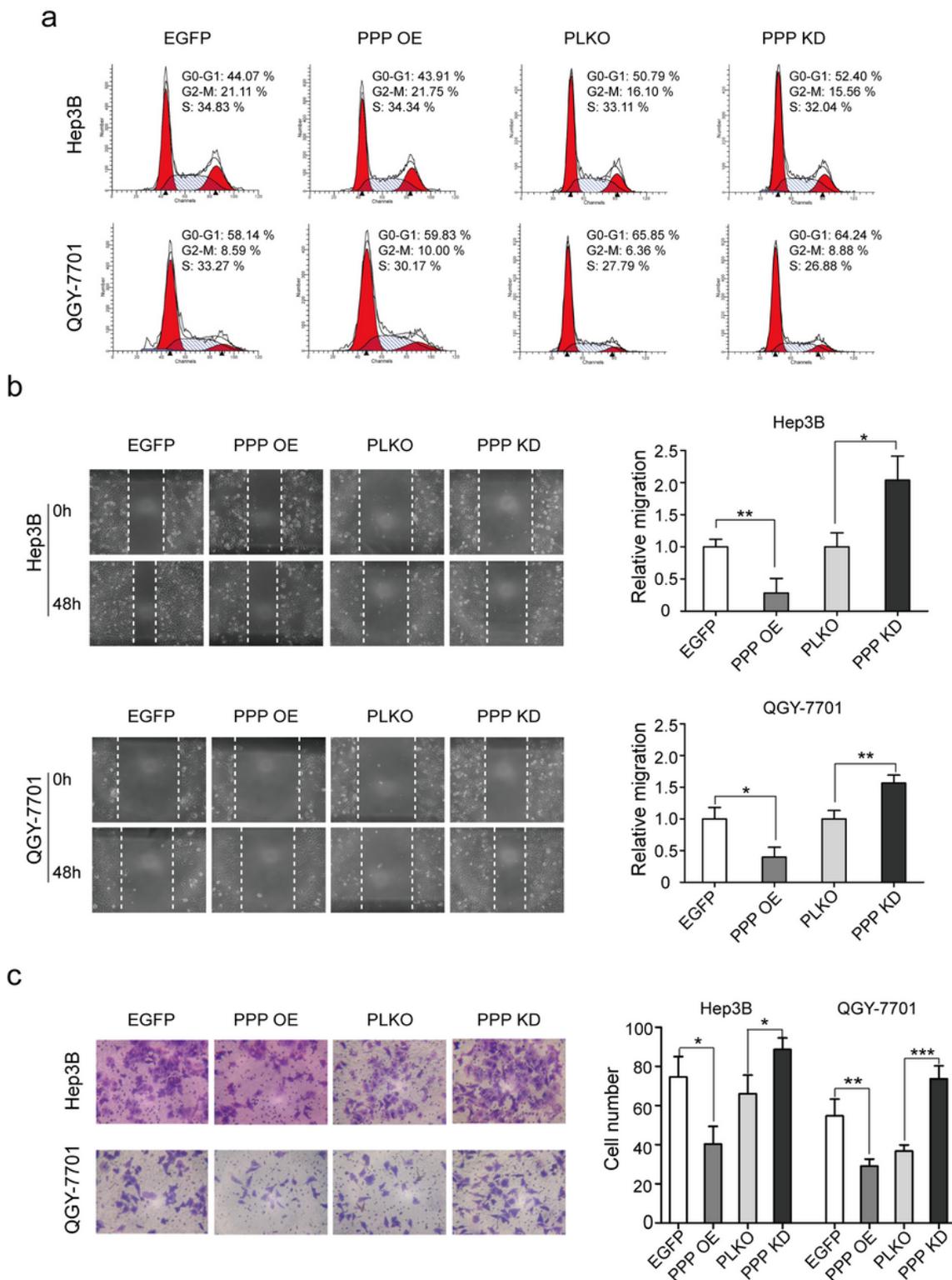


Figure 3

PPP2R5A inhibits the migration and invasion of HCC cells. a. The effect of PPP2R5A on cell cycle; b. Wound healing assay to detect the effect of PPP2R5A on cell migration, and the statistical analysis; c. Transwell assay to detect the effect of PPP2R5A on cell invasion, and the statistical analysis. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$)

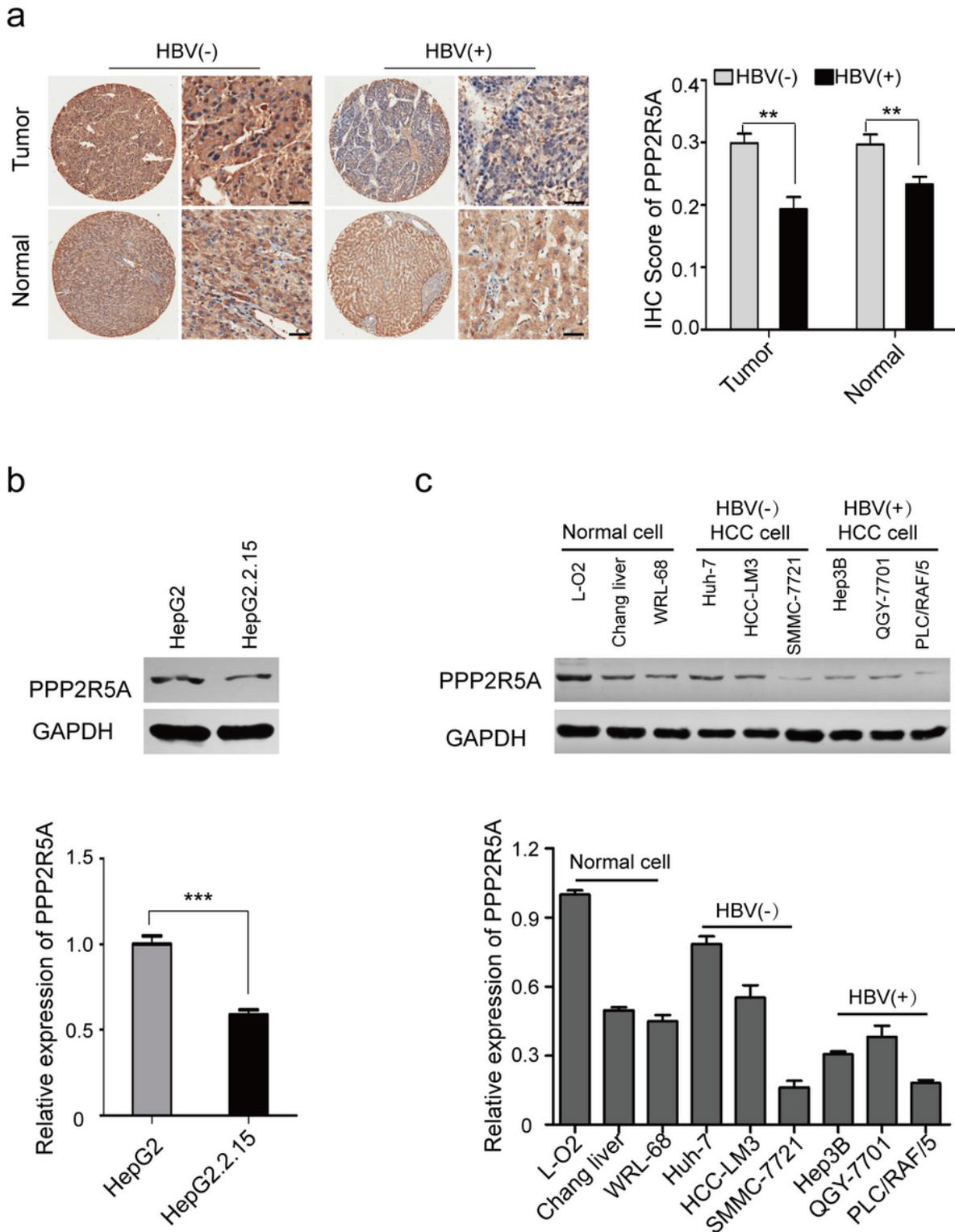


Figure 4

HBV infection leads to the down-regulation of PPP2R5A. a. The differences in the expression of PPP2R5A in liver tissues of HBV(+) and HBV(-), Bar=50 μ m; b. Western Blot of PPP2R5A in HepG2.2.15 HBV(+) and HepG2 HBV(-) HCC cells, and the bottom is the semi-quantitative statistical bar graph; c. Western Blot of PPP2R5A in HBV(+) and HBV(-) HCC cells, and the semi-quantitative statistical bar graph is shown below. (**, $p < 0.01$; ***, $p < 0.001$)

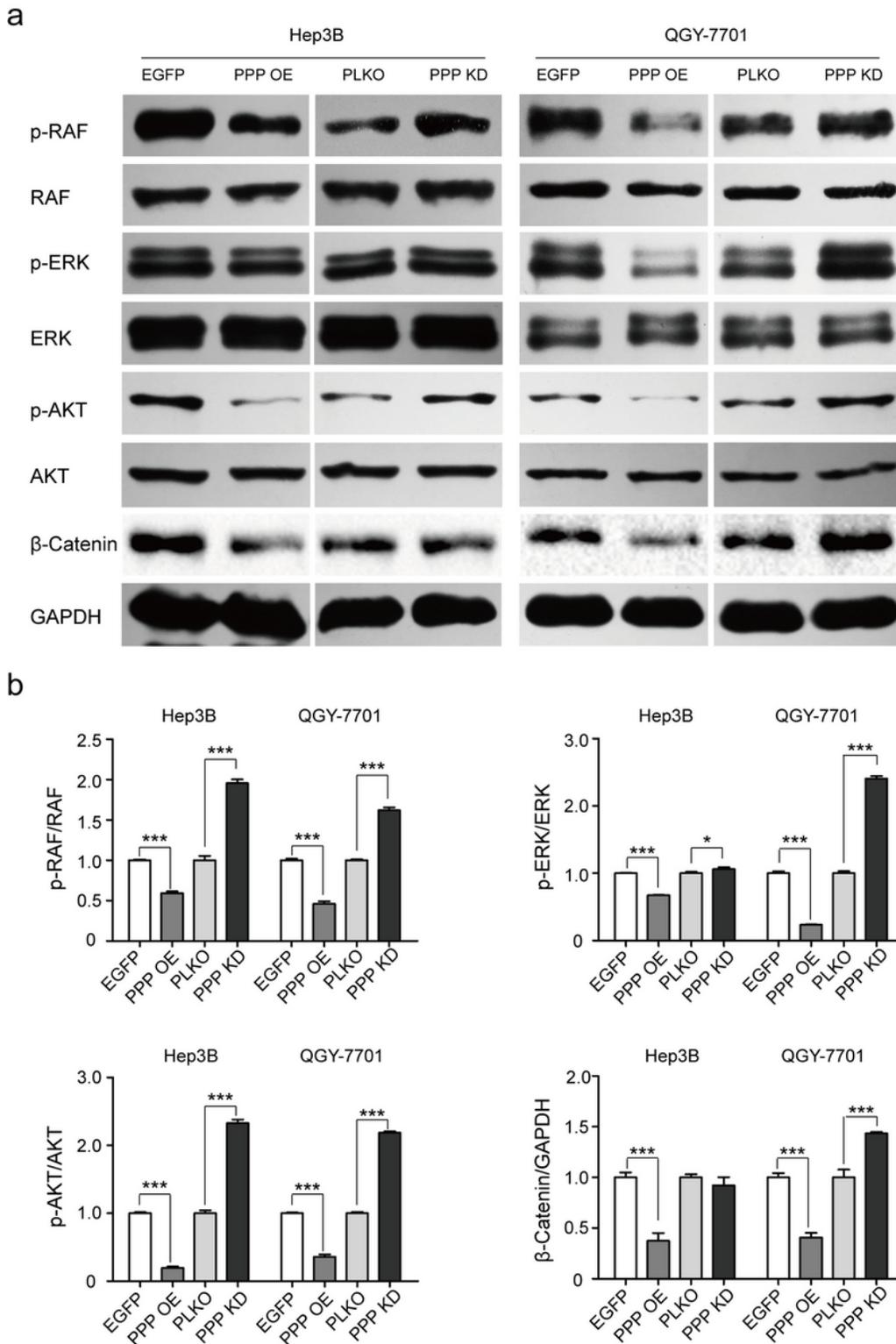


Figure 5

PPP2R5A exerts tumor suppressor efficacy by inhibiting the MAPK/AKT/WNT signaling pathway. a. Western Blot to detect the influence of PPP2R5A on the related molecules of cell signaling pathways; b. The semi-quantitative analysis of the relative changes of each molecule. (*, $p < 0.05$; ***, $p < 0.001$)

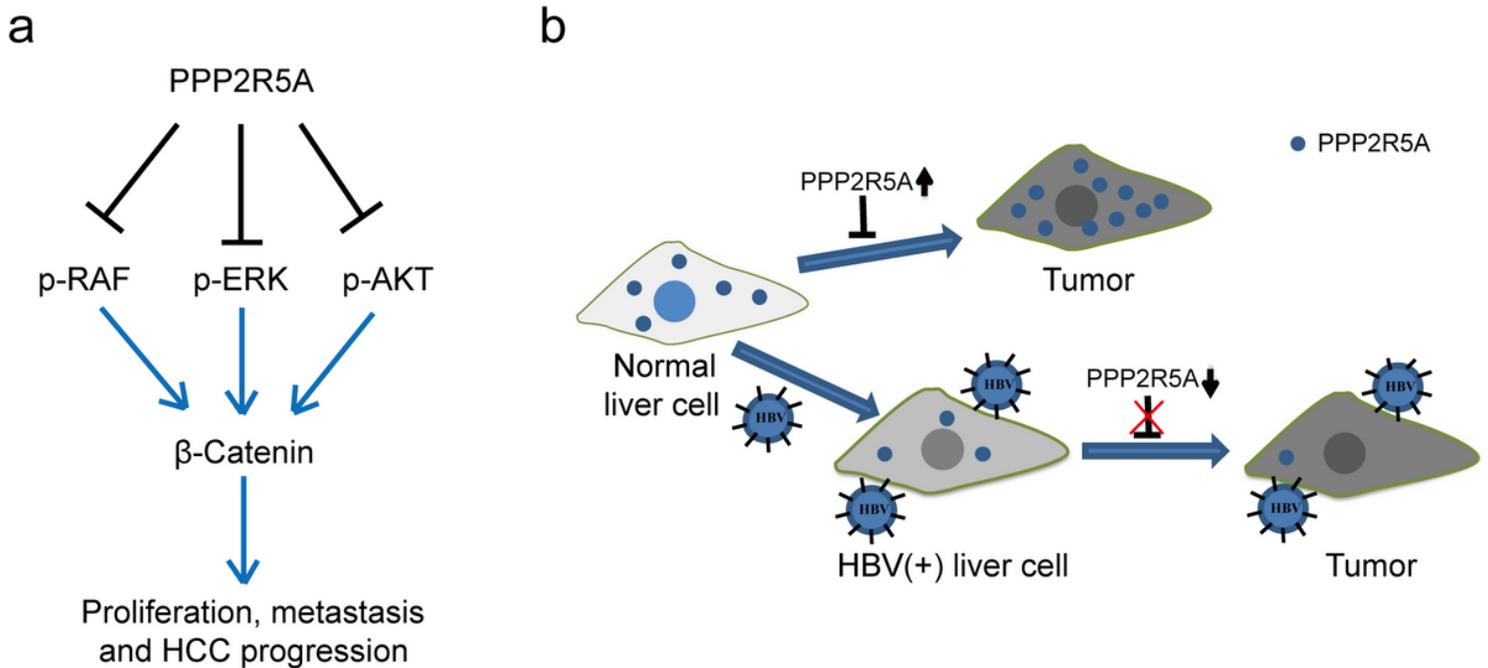


Figure 6

The molecular mechanism of HBV promoting the occurrence and development of HCC through PPP2R5A. a. PPP2R5A inhibits the activation of WNT signaling pathway by inhibiting MAPK and AKT signaling pathways. b. PPP2R5A acts as a tumor suppressor both in hepatocytes and HCC cells. Without HBV infection, when hepatocytes become cancerous, as one of the protective mechanisms, the cells will up-regulate the expression of PPP2R5A. The high expression of PPP2R5A can inhibit the proliferation and metastasis of HCC cells. When HCC patients are infected by HBV, HBV can down-regulate the content of PPP2R5A in cells. It leads to the reduction of PPP2R5A that exerts a tumor suppressor efficacy and the weakening of this cellular self-protection mechanism, thereby promoting the occurrence and development of HCC.

Supplementary Files

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