

FKBP11 upregulation promotes proliferation and migration in hepatocellular carcinoma

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Abstract.

BACKGROUND: Hepatocellular carcinoma (HCC) is one of the leading causes of cancer related deaths world over. Early diagnosis and effective treatment monitoring significantly improves patients' outcomes. FKBP11 gene is highly expressed in HCC and could play a role in its development, early diagnosis and treatment.

OBJECTIVE: This study aimed to evaluate the expression of FKBP11 in HCC, its correlation with patients' clinical characteristics and potential role in HCC development.

METHODS: Expression was determined by bioinformatics analysis, quantitative real-time PCR, western blot, and immunohistochemistry. CCK-8, Transwell and wound healing assays were used to investigate involvement in HCC development.

RESULTS: FKBP11 was significantly upregulated in HCC cells, tissues and blood (all $p < 0.001$). Its receiver operator characteristic (ROC) curve had an AUC of 0.864 (95% CI: 0.823–0.904), at a sensitivity of 0.86 and specificity of 0.78 indicating a good diagnostic potential in HCC. Its expression was markedly reduced after surgery ($p < 0.0001$), indicating a potential application in HCC treatment follow-up. Knockdown of FKBP11 in HCC cells attenuated proliferation and migration, suggesting a possible role in HCC pathogenesis.

CONCLUSION: This study thus found that FKBP11 is upregulated in HCC, and the upregulation promotes HCC development. FKBP11 levels are significantly reduced post-surgery and could be a potential diagnostic and prognostic marker for HCC.

Keywords: FKBP11, biomarker, diagnosis, hepatitis B virus, hepatocellular carcinoma

1. Introduction

World over, liver cancer is the second highest cause of cancer related deaths [1,2], with hepatocellular carcinoma (HCC) making up about 80% of the cases [3].

It is estimated that there will be approximately 1 million newly diagnosed HCC cases by 2030 [4]. Major risk factors for HCC include hepatitis B and C virus infection, liver cirrhosis and alcoholic liver disease. Chronic hepatitis B virus infection remains the major cause of HCC to date [5–7,14]. The 5-year-survival rate of HCC patients is very low ($< 12\%$), mainly due to delayed diagnosis, and factors like chemotherapy resistance [8]. This is despite the fact that surgical interventions, and early initiation on chemotherapy significantly improves patient outcomes [3]. Current di-

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agnostic methods for HCC include; computed tomography (CT), serum alpha-fetoprotein (AFP) measurement and magnetic resonance imaging (MRI). Much as they are all non-invasive, these methods combined still show low sensitivity [9]. It is thus important that newer non-invasive biomarkers having higher sensitivity and specificity are found for early diagnosis, especially in high-risk populations like those with chronic liver diseases.

FK506 binding protein 11 (FKBP11) is an enzymatic protein that catalyzes the folding of proline-containing polypeptides. Its C-terminal contains a putative transmembrane domain and a motif found within the endoplasmic reticulum (ER) membrane proteins, where it functions in protein folding and secretion [10,11]. It belongs to the FKBP family of proteins, consisting of 16 numbers that are collectively called immunophilins [10]. It modulates various kinases and cellular factors and is associated with T-cell activation, cell metabolism, cellular homeostasis, tumor carcinogenesis, and tumor progression [12]. FKBP11 mRNA is abundant in secretory tissues such as the liver and pancreas. Its gene encodes a 22 kDa pre-protein containing a 25 residue leucine-rich N-terminal leader sequence, whose cleavage leaves a 19 kDa mature protein, giving the protein its alternative name FKBP19 [13].

Recent evidences increasingly suggest that FKBP proteins regulate cell cycle, survival and apoptotic signaling pathways and influence tumorigenesis and the response to chemotherapies and radiotherapies [14,15]. Related studies in cancers other than HCC have suggested that FKBP11 promotes cell proliferation and tumorigenesis in oral squamous cell carcinoma via p53-related pathways [16], and is associated with poor prognosis in Clear-cell renal cell carcinoma [17]. In HCC, it has been found that FKBP11 is highly expressed and that it could play a significant role in HCC development [13]. In this study, we evaluated the expression level of FKBP11 in HCC, its correlation with clinicopathological characteristics of the HCC patients, and its role in the pathogenesis of HCC.

2. Materials and methods

2.1. Bioinformatics analysis of FKBP11 expression in hepatocellular carcinoma

We explored the expression of FKBP11 gene and its importance in hepatocellular carcinoma using different bioinformatics tools. We analyzed the TCGA publicly

available datasets for differential expression of FKBP11 in HCC and normal liver samples, its relative expression in different stages of HCC, race, histological types of HCC and association with hepatitis B virus infection. We then analyzed its involvement in prognosis of HCC patients using survival analysis.

2.2. Sample collection

A total of 46 HCC tissues (30 males and 16 females, mean age 57.7 ± 11.8), and their paired adjacent normal tissues from patients at Zhongnan Hospital of Wuhan University were collected from September 2020 to May 2022. Majority of the tissues were collected from HCC stage I and II patients. In addition, 167 whole blood samples were collected from January to June 2022. Ethical clearance to conduct the study was sought from Zhongnan Hospital of Wuhan University Research Ethics Review Committee (No. 2018063), and all participants provided signed informed consent. The samples were classified as; 62 from HCC patients (52 males and 10 females, mean age 55.7 ± 12.6), 40 from Hepatitis B & liver cirrhosis patients (29 males and 11 females, mean age 49.0 ± 13.3), and 65 as control, from healthy patients who came for routine physical examination; (46 males and 19 females, mean age 48.0 ± 12.9). All patients were recruited after assessing their pathology reports. Control patients were included only if they were HBsAg negative, and had no other liver diseases e.g. alcoholic, autoimmune, hepatitis C and metabolic liver disease, while HCC patients with a history of chemotherapy or radiotherapy were excluded. RNA was immediately extracted from samples, and the total RNA stored at -80°C for further processing.

2.3. RNA extraction, cDNA synthesis and quantitative real-time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from whole blood, tissues and cell cultures using the RNAeasy kits for whole blood, and tissues/cells; (Cat: R6814-02; Cat: R6812-01, Biotek Co., Ltd., China), according to the manufacturer's instructions. RNA concentration was determined using the Nano-drop 2000 spectrophotometer (Thermo Scientific Inc., Waltham, MA, USA), and then reverse transcribed into cDNA using ReverTra AceTM qPCR master mix with gDNA remover from Toyobo. (Cat: FSQ-301; Toyobo. Shanghai, China). All the cDNA samples were kept at -80°C prior to qPCR analysis. Quantitative real-time PCR was then performed using UltraSYBR Mixture real time qPCR

kit (Cat: CW0957S; CWBIOTech, China) according to the manufacturer's instructions, on the Bio-Rad CFX PCR machine. A 20- μ l total reaction volume was used consisting of 2 μ l primers, 10 μ l master mix, 6 μ l DEPC water, and 2 μ l cDNA. The cycling conditions were as follows: Initial denaturation at 95°C for 10 min; 40 cycles for denaturation at 95°C for 10 sec; annealing at 62.3°C for 30 sec, elongation at 72°C for 32 sec, and then 95°C for 15 sec, 60°C for 1 min, 95°C for 15 secs, and 60°C for 15 sec. The endogenous gene Beta actin was used as the housekeeping gene. Primers for the PCR reactions were as follows: FKBP11-Forward: 5'CTGGAGCAGAGTCTTCTCGACAT3', and FKBP11-Reverse: 5' TCCATAGGCCAAGTGAG AAGGA3'. Beta-actin-Forward: 5' GTCCACCGCAA ATGCTTCTA 3', and Beta-actin-Reverse: 5' TGCTG TCACCTTCACCGTTC 3'. All reactions were conducted in duplicates, and average Ct calculated. The double delta method was used to calculate Gene expression.

2.4. Cells culture

Hepatocellular carcinoma (HCC) cell lines; HepG2, Huh7, and Hep3B and the normal liver cell line LO2 were all purchased from Procell Inc. (Wuhan, China). All cells were cultured in DMEM medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), and 1% Penicillin-streptomycin antibiotics.

2.5. Lentivirus cell transfection

Two FKBP11 siRNAs were designed and synthesized by HanBio, Beijing China; siRNA1: GGGCAATC ATTCCTTCTCA; and siRNA2: GAGAAGCGAAGGG CAATCA. The siRNAs were constructed into a plasmid and cloned into a pHBLV lentiviral vector to generate pHBLV-FKBP11. The pHBLV-FKBP11 was then transfected into HEK 293 T cells and virus particles harvested 48 h post transfection, with the packaging plasmid psPAX2 and the envelope plasmid pMD2.G using Lipofectamine 2000 reagent (Invitrogen). Huh-7, and Hep3B cells were then infected with the recombinant lentivirus-transducing units using 1 μ g/ml polybrene (Sigma-Aldrich, Missouri, USA) following manufacturer's instructions. Transfection efficiency was ascertained using western blot after 48 hours of incubation.

2.6. Cell proliferation assay (CCK-8)

To determine cell proliferation, the Cell Counting

Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) assay was conducted. Briefly; the cells were seeded at 3×10^3 cells/well in a 96-well plate with 100 μ l complete culture medium. 10 μ l/well of CCK-8 reagent were then added to the cells at 0 h, 24 h, 48 h, and 72 h, and the plate incubated for 4 hours with the reagent at 37°C. After the 4 hours, the plate was retrieved and Optical density (OD) measured using a spectrophotometer (Spectra Max Plus, Molecular Devices, Sunnyvale, USA) at 450 nm. Measurements were repeated according to the incubation times designed above.

2.7. Cell migration assays (Transwell and scratch)

To determine cell migration ability, Transwell and scratch assays were conducted. Briefly, cells were first suspended in 200 μ l serum-free culture medium and seeded in the upper chamber of the Transwell plate (Corning, NY, USA), meanwhile in the lower chamber, a 20% FBS containing culture medium was added. The plate was then incubated for 24 h. After incubation, cells from the surface of the upper chamber were removed and those in the lower chamber stained with crystal violet. Cell quantity was then counted in five randomly selected fields under a light microscope, and the average cell amount per field determined. Second, cells were seeded in six-well plates, incubated at 37°C and 5% CO₂ and allowed to grow up to 80% confluence. Using a 200 μ l pipette tip, a straight line scratch was made in the plate, culture media changed and the cells reincubated at the same conditions as before. Cells were then observed under light microscope at 0 and 24 hours to assess the wound healing process.

2.8. Western blot analysis

Total proteins from the cells were extracted using RIPA lysis buffer (Cat: P0013; Beyotime, Shanghai, China) mixed with a protease inhibitor; PMSF (Cat: G2008; Thermo Fisher Scientific, Waltham, MA, USA). The protein concentration was measured using BCA proteins assay kit (Cat: P0010, Beyotime, Shanghai, China). Appropriate volumes equaling to 10 μ g of protein were then loaded and separated by electrophoresis on 10% SDS-PAGE gel, then transferred onto PVDF membranes (Millipore, Sigma). The membranes were blocked with 5% skimmed milk, and then incubated overnight with primary antibodies against FKBP11 (Cat: bs-13176; dilution: 1:2,000; Bioss, China), and GAPDH (Cat: K200057M; dilution: 1:5000; Proteintech, China). The blots were then incubated with HRP

secondary antibodies (Cat: AS1106; dilution: 1:6000, Aspen, China) for 1 hour at room temperature and the bands visualized using the ECL machine (Thermo Fisher Scientific, Inc.).

2.9. Immunohistochemistry analysis

Three pairs of tissues (HCC: $n = 3$, Normal-adjacent control: $n = 3$) were cut into pieces and fixed in 10% formalin for 8 hours at room temperature. They were then dehydrated in increasing concentrations of ethanol (70%, 90% and 100%) for 30 minutes each, then immersed in xylene three times for 20 minutes each. The tissues were embedded in paraffin wax at 58°C, cut into 10 μ m pieces and floated in a water bath at 56°C, then mounted onto slides. Once mounted on the slides, the tissue sections were re-immersed in xylene, twice for 20 minutes each, then rehydrated by immersion in decreasing concentrations of ethanol (100%, 95%, 70% and 50%) for 5 minutes each then rinsed in deionized water and rehydrated in wash buffer for 10 minutes. Endogenous peroxidase activity was blocked by incubating in 3% H₂O₂ solution, and the tissue sections blocked with goat serum for 30 min and incubated with FKBP11 antibody (Cat: bs-13176; dilution: 1:100, Bioss, China) overnight at 4°C. Subsequently, samples were incubated with the corresponding HRP-conjugated secondary antibody solution (Cat: AS1106; dilution: 1:100) for 45 min. DAB (Cat: ab64328; Abcam, USA) substrate solution was then applied for antibody staining for 10 min. Nuclei were stained with hematoxylin. Cover slips were then applied and the sections visualized under light microscope.

2.10. Statistical analysis

Normally distributed data were presented as mean $\pm$ standard deviation (SD) and skewed data as median and inter-quartile ranges. The Shapiro-Wilk and Kolmogorov-Smirnov tests were conducted to check the normality of distributions. For the paired tissue samples, the Wilcoxon matched-pairs signed rank test was used, while the Mann-Whitney test was used for the non-paired blood samples. The Kruskal-Wallis variance analysis was performed for more than two groups while, qualitative data was tested using Chi-square test. Statistical data analysis were performed in SPSS 17.0 software (SPSS, Chicago, IL, USA) and GraphPad Prism 8.0.2 software (GraphPad software, La Jolla, CA, USA). IHC results were analyzed using Image J software to quantify the staining intensity [18]. All statistical tests were two-sided and significance level set at $P < 0.05$.

3. Results

3.1. Bioinformatics analysis of FKBP11 expression in Hepatocellular carcinoma

3.1.1. Expression

First, we explored the mRNA expression of FKBP11 in hepatocellular carcinoma (HCC) and normal liver samples using the publicly available TCGA data sets. GEPIA2 (<http://gepia2.cancer-pku.cn>), OncoDB https://oncodb.org/genomic_profile_express_non_virus.html and the Kaplan-Meier plotter https://kmplot.com/analysis/index.php?p=service&cancer=liver_rnaseq were used. FKBP11 was assessed for differential expression in HCC, expression in HCC patients with hepatitis B virus infection, and association with clinical characteristics of the patients. Survival analysis was then conducted for FKBP11 expression and the different categories of patient characteristics. First, analysis of FKBP11 expression in HCC revealed that it could be a pan cancer gene; significantly upregulated in several cancers including HCC, (Fig. 1A and B). Expression by patients' clinical characteristics indicated that it is differentially expressed according to race, histology and the pathological M stage (Fig. 1C–E).

3.1.2. Survival analysis

Survival analysis revealed that there is no statistically significant difference in overall survival (OS) between HCC patients with high FKBP11 expression and those with low expression, however, recurrence free survival (RFS), progression free survival (PFS) and disease specific survival (DSS) were significantly different among the different clinical characteristics of patient's with high and low FKBP11 expression as follows: White people with low FKBP11 expression had better RFS ($p = 0.0064$), PFS ($p = 0.0097$), and DSS ($p = 0.024$) than those with high expression (Fig. 2A–C). Among alcoholic HCC patients, OS was better in those who consumed less alcohol ($p = 0.047$) (Fig. 2D). By gender, RFS was better among males with low FKBP11 expression ($p = 0.043$), (Fig. 2E) while the opposite was true among females; where those with high FKBP11 expression had better survival ($p = 0.021$) (Fig. 2F).

3.2. Association with Hepatitis B virus infection

We then explored the association between FKBP11 expression and hepatitis B virus (HepBV) infection in HCC patients with HepBV. Here, the analysis showed that FKBP11 was significantly upregulated in HCC

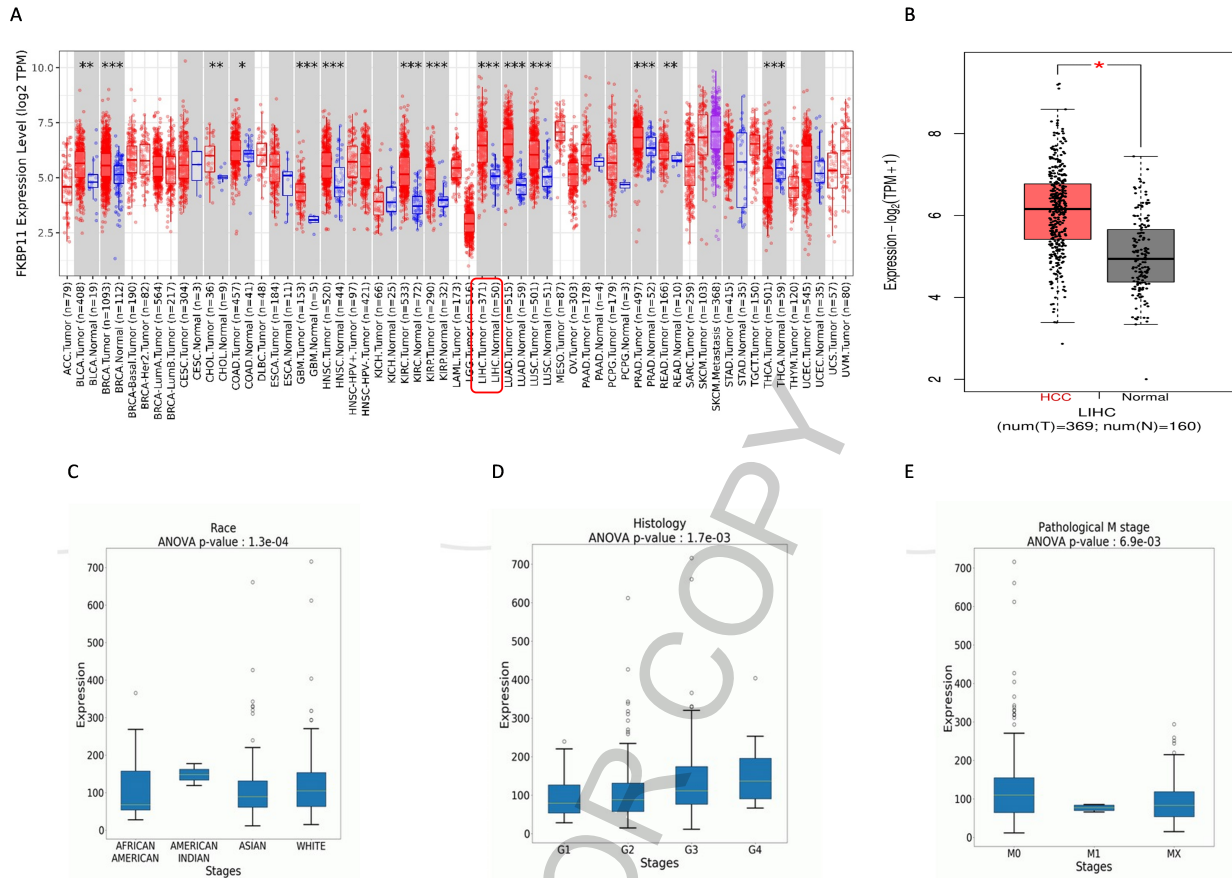


Fig. 1. Bioinformatics analysis of FKBP11 expression using the TCGA datasets GEPIA2 and OncoDB. A: FKBP11 is a pan cancer gene whose expression is upregulated in various cancers, including HCC. B: differential expression of FKBP11 mRNA in HCC versus normal samples ($*p < 0.05$). C: FKBP11 expression in HCC by race (ANOVA $***p = 0.00013$). D: FKBP11 expression in HCC by tumor histology (ANOVA $**p < 0.0017$). FKBP11 expression in HCC by tumor stage (ANOVA $**p < 0.0069$).

patients with HepBV infection than those without (Fig. 3A). Survival analysis showed that although HCC patients with HepBV and low expression of FKBP11 had better OS, the difference was not statistically significant ($p > 0.05$) (Fig. 3B), while HCC patients without HepBV in whom FKBP11 expression was high had worse OS ($p = 0.043$) (Fig. 3C).

3.3. FKBP11 is upregulated in HCC tissues and cell lines

To confirm the bioinformatics results, quantitative real time PCR, western blot and immunohistochemistry were used to determine the differential expression of FKBP11 in HCC tissues, cell lines, and their corresponding normal controls. Here, FKBP 11 exhibited significantly high expression levels in HCC tissues compared to their adjacent normal controls, (Fig. 4A; $p < 0.01$). Waterfalls plot demonstrated that FKBP11

was upregulated by at least twofold in 19.5% (9/46) of the HCC tissues on a log scale (Fig. 4B). These results were corroborated by immunohistochemistry where HCC tissue stained strongly with anti-FKBP11 antibody compared to normal liver tissue (Fig. 4C, D and G, $P < 0.05$). Similarly, FKBP11 mRNA (Fig. 4E) and proteins (Fig. 4F and H), were upregulated in all the HCC cell lines (HepG2, Hep2B and HuH7A) compared to the normal liver cell line (L02).

3.4. Clinicopathological data analysis of the HCC tissues

Details of the patients' associated clinical parameters of all the 46 HCC tissues were analyzed and summarized in Table 1. Of the 46 patients 30 were male and 16 female. 28 patients were above the age of 55. In terms of tumor characteristics, 44 belonged to TNM I~II while 2 belonged to TNM III. 36 patients had tumor size bigger

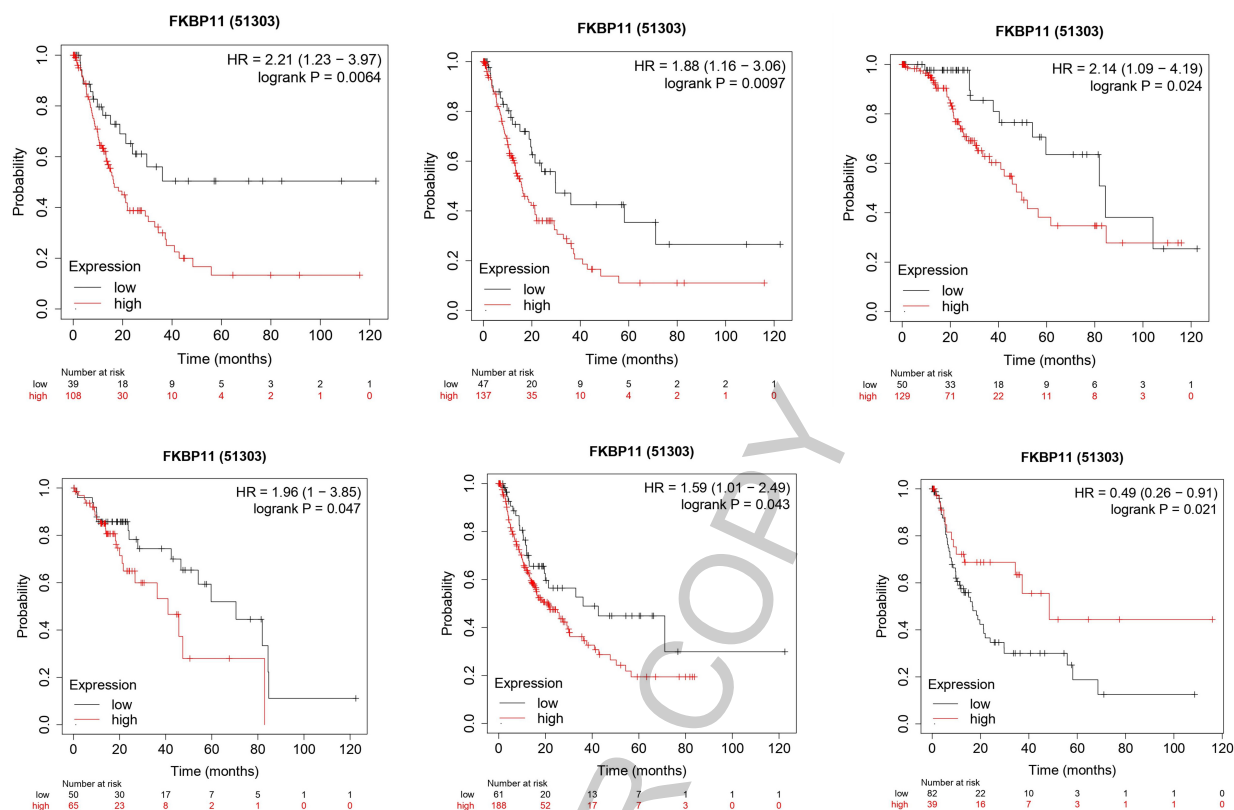


Fig. 2. Kaplan-Meier survival analysis for race, alcohol consumption, and gender. A: Recurrence free survival (RFS) among white people. Those with low FKBP11 expression had better outcomes than those with high expression (** $p = 0.0064$). B: Progression free survival (PFS) among white people. Those with low FKBP11 expression had better outcomes than those with high expression (** $p = 0.0097$). C: Disease specific survival (DSS) among white people. Those with low FKBP11 expression had better outcomes than those with high expression (* $p = 0.024$). D: Overall survival among HCC patients who were alcoholics. Those with low alcohol consumption had better OS than those with high alcohol consumption (* $p = 0.047$). E: Recurrence free survival among male HCC patients. Those with low FKBP11 expression had better survival (* $p = 0.043$). F: Recurrence free survival among female HCC patients. Those with high FKBP11 expression had better survival (* $p = 0.021$).

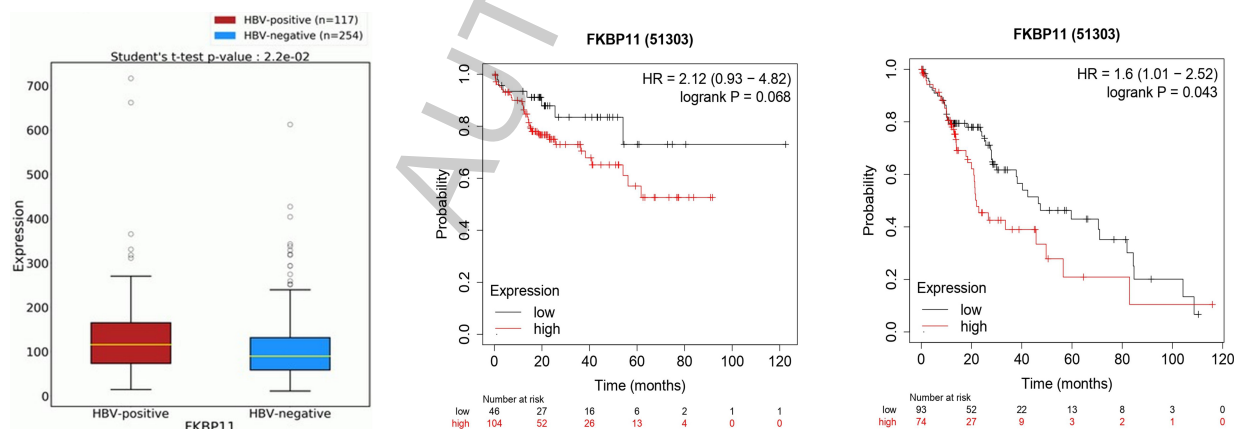


Fig. 3. Expression of FKBP11 in hepatitis B positive and negative HCC patients and their survival analysis graphs. A: FKBP11 was upregulated in HCC patients with hepatitis B virus infection than in those who were negative for the virus (* $p = 0.022$). B: Although HCC patients with hepatitis B infection and low expression of FKBP11 had better overall survival (OS) than those with low expression of FKBP11, the difference was not statistically significant ($p > 0.05$). C: HCC patients without hepatitis B virus infection in whom FKBP11 expression was high had worse overall survival than those in whom FKBP11 expression was low (* $p = 0.043$).

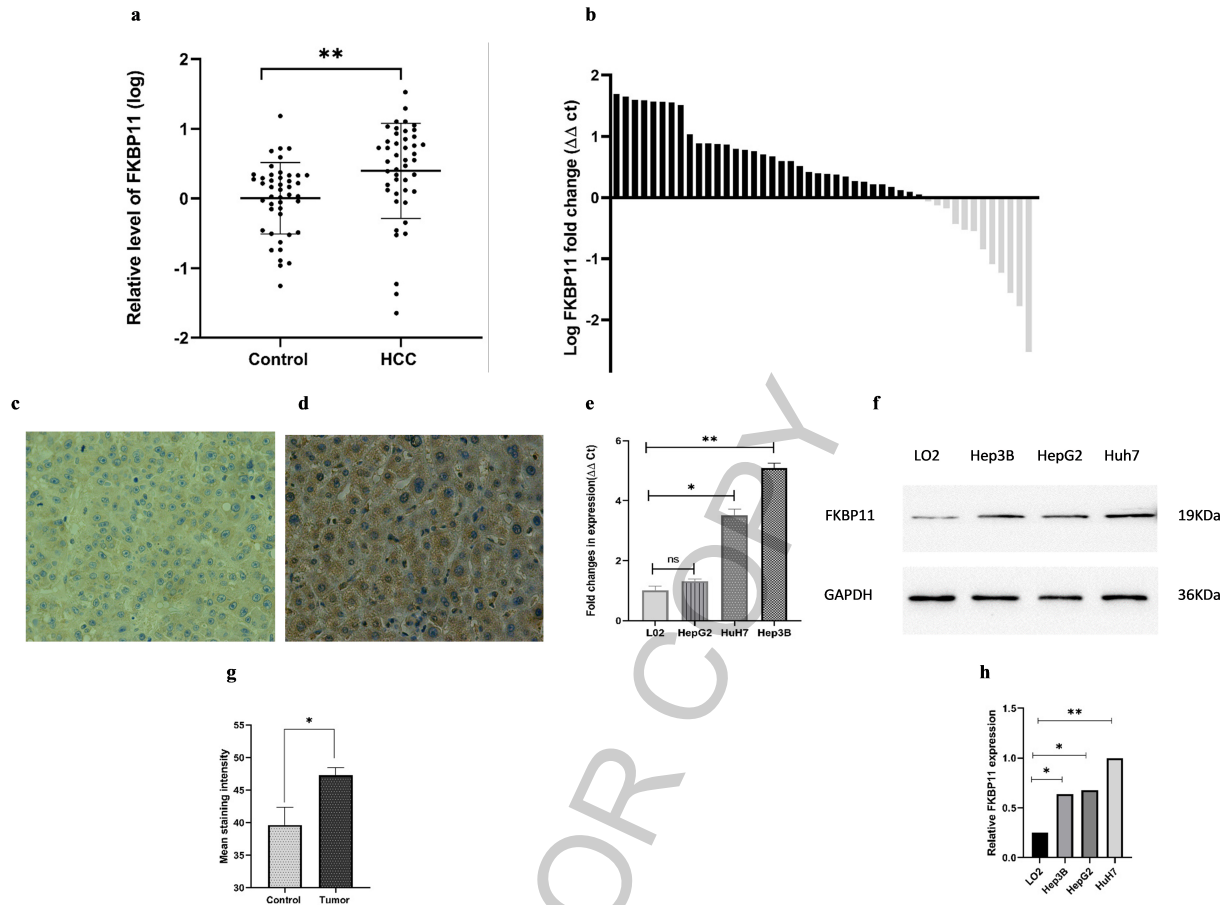


Fig. 4. Expression of FKBP11 gene in HCC. A: FKBP11 is upregulated in HCC tissues compared to normal adjacent cancer tissues, dependent *t*-test (** $p < 0.01$). B: Waterfalls plot indicating FKBP11 expression in HCC tissues and controls. C, D and G: IHC result of FKBP11 in normal liver tissue and HCC tissue, ($P < 0.05$). E: FKBP11 is upregulated in HCC cell lines. Kruskal-Wallis test with Dunn's post hoc correction (** $p < 0.001$). F and H: Western blot analysis of FKBP11 expression in cell lines (* $P < 0.05$).

than 5 cm. 22 patients had GGT levels greater than normal while 14 had AFP greater than 200 ng/ml. 45.6% of the patients were regular smokers while 52.1% were regular alcohol consumers. FKBP11 upregulation was not associated with any particular clinical characteristics of the patients. Although expression levels varied among the different variables, none was statistically significant, probably due to the small number of samples analyzed.

3.5. FKBP11 level in peripheral blood

We then explored the level of expression of FKBP11 in peripheral blood of 62 HCC patients, 40 liver cirrhosis patients, and 65 healthy control cases. Demographic and clinical characteristics of the patients are presented in Table 2. No significant difference was observed in gender as a risk factor, however, significant

differences were found in age, ALT, AST, GGT, TBIL, DBIL and TP. As shown in Fig. 5A, the expression levels of FKBP11 differed significantly between HCC and cirrhosis ($P < 0.05$), and controls ($P < 0.0001$), and between cirrhosis and controls ($P < 0.0001$). FKBP11 expression was higher in HCC than both cirrhosis and controls. To determine FKBP11 potential as a diagnostic biomarker for HCC, Receiver Operator Characteristic (ROC) curve was plotted to show its diagnostic characteristics. The results showed that FKBP11 could be a potential diagnostic marker for HCC with an AUC of 0.864 (95% CI: 0.823–0.904), sensitivity of 0.86 and specificity of 0.78 (Fig. 5B).

3.6. FKBP11 expression was downregulated post-surgery

Next, we evaluated whether peripheral blood ex-

Table 1
Clinicopathological relevance analysis of FKBP11 in HCC patients

Characteristics	n	FKBP11 relative expression (mean $\pm$ SD)	Significance (P)
Tissue			
HCC	46	3.23 $\pm$ 2.36	
Gender			0.82
Male	30	3.1 $\pm$ 1.7	
Female	16	3.4 $\pm$ 3.2	
Age			0.62
< 55	18	3.4 $\pm$ 2.9	
$\geq$ 55	28	3.1 $\pm$ 1.9	
Tumor size			0.22
< 5 cm	10	2.5 $\pm$ 2	
$\geq$ 5 cm	36	3.4 $\pm$ 2.4	
TNM stage			0.81
I~II	44	3.2 $\pm$ 2.4	
III~IV	2	3.2 $\pm$ 1.8	
Alcoholism			0.21
Yes	24	2.8 $\pm$ 1.8	
No	22	3.7 $\pm$ 2.7	
Smoking			0.36
Yes	21	2.8 $\pm$ 1.9	
No	25	3.5 $\pm$ 2.6	
AST			0.42
< 40	34	3.1 $\pm$ 2.5	
$\geq$ 40	12	3.4 $\pm$ 1.9	
ALT			0.63
< 50	44	3.1 $\pm$ 2.3	
$\geq$ 50	2	3.6 $\pm$ 2.6	
GGT			0.307
< 55	24	3.6 $\pm$ 2.8	
$\geq$ 55	22	2.7 $\pm$ 1.6	
HepBV status			0.031*
Positive	17	3.2 $\pm$ 2.4	
Negative	29	1.2 $\pm$ 1.6	
AFP			0.98
< 200	32	3.1 $\pm$ 1.9	
$\geq$ 200	14	3.5 $\pm$ 3.1	

Key. Data presented as mean and corresponding standard deviations. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma-glutamyl transferase. AFP: Alpha-feto protein.

pression level of FKBP11 could be used to monitor treatment dynamics. We examined the expression of FKBP11 in 14 pairs of plasma samples collected from the same patients before and approximately two weeks after surgery. Interesting, FKBP11 was down-regulated in 71.4% (10/14) of the samples, indicating that FKBP11 could be useful in monitoring treatment progress in HCC (Fig. 5C).

3.7. FKBP11 knockdown inhibits HCC cell proliferation, and migration

After demonstrating cellular expression, we then determined the role of FKBP11 in HCC progression. We used siRNA to knock down FKBP11 in both Hep3B

and HuH7 cells. Transfection efficacy was confirmed by western blot analysis (Fig. 6A). CCK-8 assay showed that compared to the control group, proliferation of the si-FKBP11 transfected cells was significantly inhibited. The degree of inhibition became pronounced as culture time extended, stagnating at around 72 h (Fig. 6B and C). Similarly, si-FKBP11 transfected cells manifested significantly reduced ability to migrate compared to si-NC cells in Transwell and wound healing assays, (Fig. 6D–K, respectively. All $p < 0.05$).

4. Discussion

Hepatocellular carcinoma remains a major public health concern to date, owing to its high incidence, often late diagnosis and poor prognosis [19,20]. According to reports, persistent hepatitis B infection (HBV) is directly responsible for more than 50% of the cases, making HCC a major burden in HBV endemic regions of the world [21]. To improve early diagnosis and prognosis, it's vital that new biomarkers are identified, since AFP that is the most widely used biomarker to date shows limited sensitivity and specificity [22,23].

FK506 binding protein 11 (FKBP11) is an enzymatic protein that catalyzes the folding of proline-containing polypeptides [10]. Recent evidence suggest that it regulates cell cycle, survival and apoptotic signaling pathways and influence tumorigenesis and the response to chemotherapies and radiotherapies [12]. Furthermore, it is highly expressed in hepatocellular carcinoma, suggesting that it could play a specific role in the development of hepatocellular carcinoma [13].

In this study, we assessed the expression of FKBP11 in hepatocellular carcinoma and its potential utility as a marker of prognosis and treatment monitoring. The study demonstrated that FKBP11 is significantly upregulated in hepatocellular carcinoma patients compared to controls. Analysis of the association between its up-regulation in HCC tissues and clinical characteristics of the tissue providers showed that its expression was significantly associated with HepBV infection. None of the patients in this study had information on hepatitis C virus infection, so we couldn't examine its association with hepatitis C. From the bioinformatics analysis, HCC patients with HepBV infection and high expression of FKBP11 had worse survival outcomes than those with low expression. According to Peneau et al. [24], HepBV integration into liver cells led to the distant alteration of cancer driver genes such as TERT, TP53 and MYC resulting into HCC with very poor prognosis. Whether

Table 2
Demographic and clinical characteristics of the patients

Characteristics	HCC N = 61	Cirrhosis N = 40	Control N = 65	P value
Gender				0.871 ^a
Male	51	29	46	
Female	10	11	19	
Age				0.007 ^a
< 55	25	23	42	
≥ 55	36	17	23	
ALT	35 (20.8, 75.8)***	26 (19.5, 78)**	23.1 (18, 25.6)	< 0.0001 ^b
AST	42.5 (24.8, 86.5)***	34 (26.5, 75)***	19 (14, 24)	< 0.0001 ^b
GGT	81.5 (46.3, 152)**	58 (22, 175) ^{ns}	31.3 (16.5, 41.5)	0.0028 ^b
TBIL	19.9 (13.7, 29.9)***	24.1 (17.7, 39.2)***	12.8 (10.4, 16.4)	< 0.0001 ^b
DBIL	5.3 (3.08, 8.6)***	7.9 (4.9, 14.7)***	2.4 (1.8, 3.55)	< 0.0001 ^b

Data are presented as Median (25 Percentiles, 75 Percentiles). ** $P = 0.0028$, *** $P < 0.0001$; ns: Non-significant vs Control. ^aChi-square test; ^bKruskal-Wallis. Abbreviation: ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBil: Total bilirubin, DBil: Direct bilirubin, GGT, γ -glutamyl transferase.

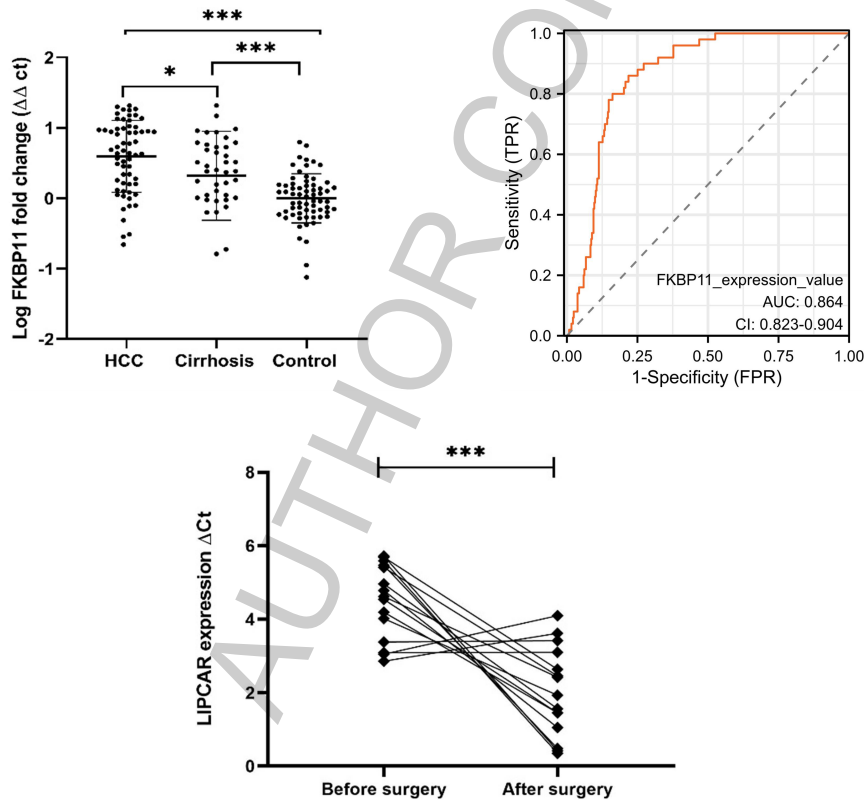


Fig. 5. FKBP11 in peripheral blood. A: Expression levels in HCC and liver cirrhosis vs control, Kruskal-Wallis test, and Dunn's post-hoc analysis (*** $P < 0.0001$). B: ROC curve showing diagnostic ability of FKBP11 in HCC. C: Pre and post-surgery levels of FKBP11 in 14 pairs of blood samples from same patients, (*** $p < 0.0001$).

this scenario could be true for FKBP11 needs to be studied.

From the peripheral blood samples, there was no difference in gender, smoking and drinking as risk factors among the subject groups, however age, (> 55 years),

liver function enzymes; ALT, AST, GGT, and other liver function biomarkers; TBIL, DBIL and TP were significantly different. Ideally, a good biomarker should be easy to access and be as less invasive as possible. In this respect, we assessed the expression of FKBP11

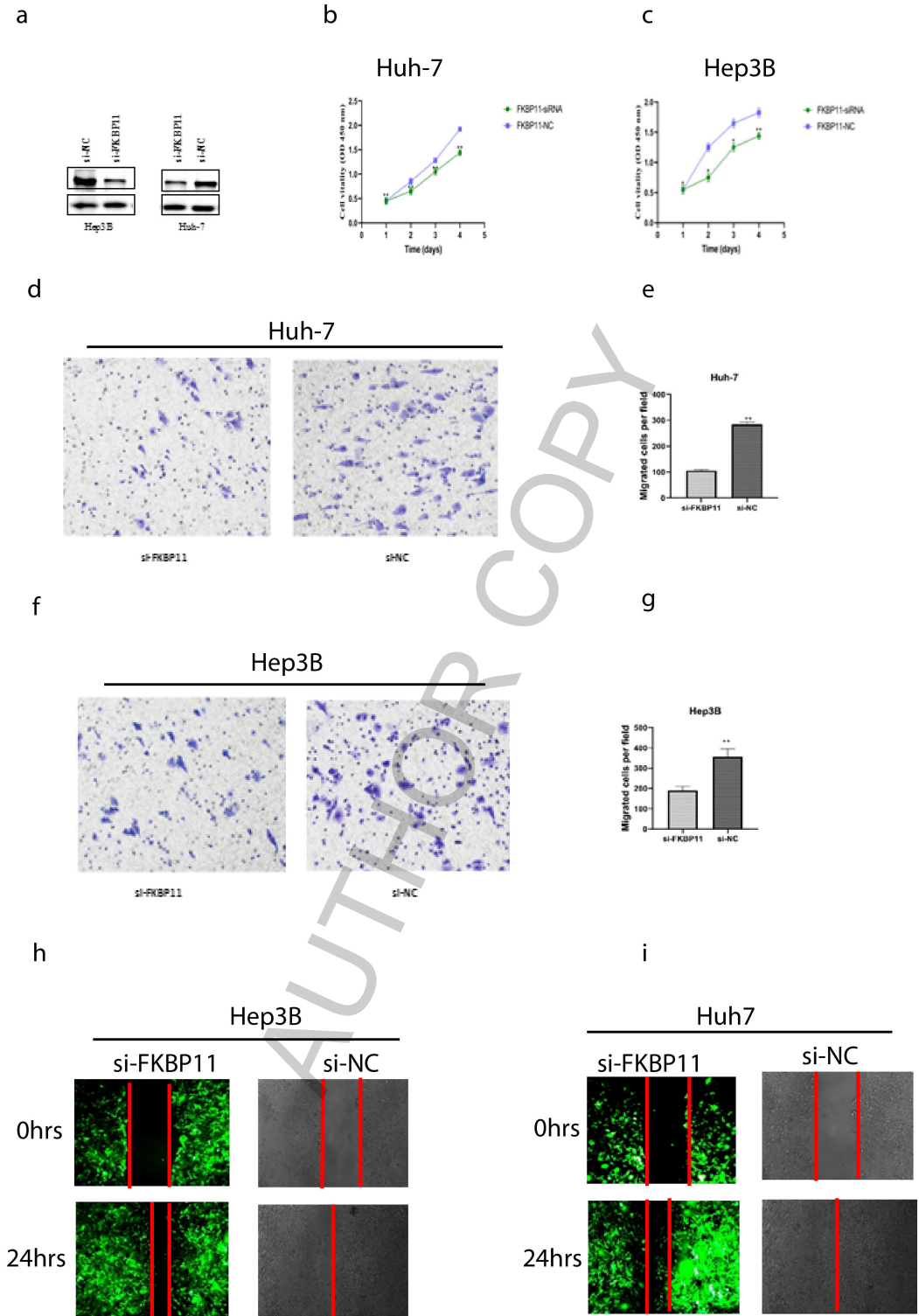


Fig. 6. Proliferation and migration assays. A: siRNA knockdown of FKBP11 in Hep3B and Huh-7 cells. B and C: CCK-8 assay results. D–F: Transwell assay results in Huh-7 cells, ($p < 0.05$). G–I: Transwell assay results in Hep3B cells, ($p < 0.05$). J: Wound healing assay in Hep3B cells. K: Wound healing assay in Huh-7 cells.

in peripheral blood leukocytes. Just like in the tissues and cultured cells, expression was upregulated in blood, and ROC curve analysis showed that it had a good diagnostic potential for HCC with an AUC of 0.864 (95% CI: 0.823–0.904). Further examination of expression in paired pre and post-surgery samples revealed a significantly downregulated expression, indicating that FKBP11 could also be potentially useful in monitoring treatment progress. These findings need to be validated in larger studies.

Our study is in agreement with a study by Lin et al. [13] who showed that FKBP11 was upregulated in hepatocellular carcinoma. However, Lin et al. only demonstrated expression in cancer tissues unlike ours which also involved cultured cells and peripheral blood leukocytes. Other cancer related studies have shown that FKBP11 promotes cell proliferation and tumorigenesis in oral squamous cell carcinoma via p53-related pathways [16], and is associated with poor prognosis in Clear-cell renal cell carcinoma [17]. In this regard, we explored the potential role of FKBP11 in HCC pathogenesis. Knock down of FKBP11 in HCC cell lines (Hep3B and HuH-7) significantly reduced proliferation and migration of the cells *in vitro*. These results suggest that FKBP11 could be involved in promoting HCC development. Whether it is involved in HCC metastasis or not, and its molecular mechanism of involvement are areas of future studies.

5. Limitations

Our study had the following limitations;

First, although we demonstrated the upregulation of FKBP11 in HBV associated hepatocellular carcinoma, and its promotion of HCC, the exact mechanism by which it promotes the cancer is still unknown. This is a likely area of research in our future studies.

Second, downregulated expression after surgery does not necessarily mean it's applicable for prognosis prediction and treatment monitoring. To confirm this a long follow-up study is required where the reduction in expression can be correlated with patients' survival outcomes and other factors associated with HCC prognosis.

6. Conclusion

In summary, FKBP11 expression was upregulated in peripheral blood, cultured cells and hepatocellular

carcinoma tissues compared to controls, and was significantly associated with hepatitis B virus infection. Its differential expression in HCC patients versus normal individuals has potential for diagnostic application in HCC, and monitoring of treatment progress. Lastly, Knockdown of FKBP11 in HCC cells significantly reduced cell proliferation and migration, suggesting a possible role in promoting HCC development.

Author contributions

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Institutional review board statement

Ethical approval was sought from the Zhongnan Hospital of Wuhan University Research Ethics Committee, number 2018063. All participants provided signed informed consent to participate.

Data availability statement

All the data involved in this study are with the corresponding author and shall be made readily available upon request.

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Conflict of interest

The authors declare no conflict of interest.

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