# Identification of FKBP11 as a Biomarker for Hepatocellular Carcinoma

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**Abstract.** *Background: Glycine N-methyltransferase (GNMT) is a tumor suppressor of hepatocellular carcinoma (HCC). High proportions of GNMT knockout mice developed HCC. We previously identified a potential novel marker from Gnmt knockout mice, FK506 binding protein 11 (FKBP11). Here, we determined the clinical usefulness of FKBP11. Patients and Methods: FKBP11 expression levels were analyzed in 123 paired tumor and tumor-adjacent non-tumorous (TA) tissue samples from patients with HCC and in 29 benign liver samples from patients with hemangioma using quantitative real-time polymerase-chain-reaction. Results: FKBP11 was expressed at a higher level in tumor tissues compared to TA tissues ($p<0.01$). Moreover, we observed a significantly higher level of FKBP11 in TA tissues than in benign liver samples ($p<0.01$). Interestingly, expression of FKBP11 was higher in hepatitis viral-infected TA and benign tissues than in samples without viral etiology ($p<0.05$). Conclusion: These results suggest a progressively elevated expression of FKBP11 during the development of HCC and FKBP11 has the potential to be an early marker for HCC.*

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Hepatocellular carcinoma (HCC) is the third leading cause of cancer deaths worldwide (1, 2). Treatment options for the management of HCC are extremely limited. Only a small proportion of patients are eligible for surgical management involving hepatic resection or transplantation (3). Other therapies, such as radiofrequency ablation and transarterial chemoembolization (TACE), are often used basically for palliation (4). The application of the multitargeted kinase inhibitor sorafenib in the clinical management of patients with HCC represents a breakthrough in translational medicine (2, 5). However, its benefits are modest and occur only in selective patients (2). Thus, early detection of HCC is generally considered to be crucial in improving outcome.

Glycine *N*-methyltransferase (GNMT) is a tumor suppressor of HCC (6, 7). It regulates the ratio of *S*-adenosylmethionine to *S*-adenosylhomocysteine and serves as a folate-binding protein (8, 9). In addition, GNMT binds carcinogens such as polyaromatic hydrocarbons and aflatoxins; and prevents the DNA adduct formation and cytotoxicity induced by these carcinogens (10-12). Diminished levels of GNMT were observed in both human HCC cell lines and tumor tissues (13, 14). Previously, we and another research team reported that *Gnmt*$^{-/-}$ mice of both genders develop HCC spontaneously at high rates and the pathology of the liver tumors mimics multiple stages of human HCC development, including chronic hepatitis, fatty nodules, hemangioma, dysplastic nodules and HCC (6, 7). Therefore, *Gnmt*$^{-/-}$ mice could serve as a good model for identification of novel early HCC markers. By utilizing this model, we identified FK506 binding protein 11 (FKBP11) as a potential early HCC marker.

Table I. *Main clinical and histopathologic features of 123 patients with hepatocellular carcinoma.*

| Clinicopathological feature | Variable | No. of cases (%) |
|---|---|---|
| Mean age±SD, years (range)] | 60.8±12.3 (12-86) | 123 (100) |
| Gender | male | 83 (67.5) |
| | female | 40 (32.5) |
| Viral infection | NBNC | 15 (12.2) |
| | HBV | 51 (41.5) |
| | HCV | 57 (46.3) |
| Cirrhosis | No | 58 (47.2) |
| | Yes | 65 (52.8) |
| TNM stage (AJCC and UICC, 7th ed.) | I | 60 (48.8) |
| | II | 38 (30.9) |
| | IIIA | 17 (13.8) |
| | IIIB | 5 (4.1) |
| | IIIC | 2 (1.6) |
| | IVA | 1 (0.8) |
| Tumor type | Solitary | 92 (74.8) |
| | Multiple | 31 (25.2) |
| Vascular invasion | Absent | 72 (58.5) |
| | Venous invasion or vein tumor thrombosis | 51 (41.5) |

HBV, patients with hepatitis B virus surface antigen (HBs Ag); HCV, patients with antibody to HCV; NBNC, patients without HBs Ag and antibody to HCV.

FKBPs are peptidyl-prolyl *cis/trans* isomerases (PPIases) that bind immunosuppressive drugs such as FK506, cyclosporin, and rapamycin, and are also known as immunophilins (15). FKBP12, the most abundant member of this family, can form a complex with rapamycin. The FKBP12/rapamycin complex blocks translation *via* inhibition of target of rapamycin (TOR) kinase (16). Many members of the immunophilins family have been implicated in events such as protein folding, assembly, and trafficking; co-regulation of molecular complexes; transcriptional and translational regulation; and cell–cell interactions (17). FKBP11 is a novel member and was first described by Rulten and colleagues (15). *FKBP11* mRNA is abundant in secretory tissues such as liver and pancreas. *FKBP11* gene encodes a 22 kDa pre-protein with a leucine-rich N-terminal leader sequence of 25 residues. Cleavage of the leader peptide would leave a 19 kDa mature protein, thus FKBP11 is also named FKBP19. The C-terminal of this protein contains a putative transmembrane domain followed by a motif found in endoplasmic reticulum (ER) membrane proteins. Thus, FKBP11 has been proposed to be involved in protein folding and secretion (15, 18).

In this study, the expression of the *FKBP11* gene was examined in tumor and the surrounding non-tumorous liver tissue collected from 123 patients with HCC; and the correlation between the FKBP11 expression and the clinicopathological findings were evaluated.

## Patients and Methods

*Patients and tissue specimens*. RNA specimens were obtained from the following two groups of patients through the Taiwan Liver Cancer Network (TLCN, http://140.112.133.121/index.action): a) tumor and tumor-adjacent tissue pairs from 123 HCC patients (Table I) and b) normal liver tissues from 29 patients with liver hemangioma. Five medical centers; National Taiwan University Hospital, Chang-Gung Memorial Hospitals at Linko and Kaohsiung, and Veterans General Hospitals at Taichung and Kaohsiung, participate in the TLCN. Preoperative written informed consents were obtained from all the patients recruited by the TLCN. We made two applications to the TLCN. In the first application for specimens, we requested RNA from 60 patients with HCC and RNA samples of benign liver tissue from 30 hemangioma patients. The 60 patients were subcategorized into three groups: 20 patients (10 males and 10 females) were hepatitis B virus surface antigen (HBs Ag)-positive (HBV group), 20 patients (10 males and 10 females) were anti-hepatitis C virus (HCV) antibody-positive (HCV group), and 20 patients (10 males and 10 females) were negative for both HBs Ag and anti-HCV antibody (NBNC group). One female patient negative for HBs Ag and anti-HCV antibody was excluded due to misdiagnosis. In the second application, we obtained RNA samples from 80 patients with HCC and categorized the samples into early-stage (TNM stage I, n=40), and late-stage (beyond TNM stage I, n=40). Since we excluded 16 patients who had received other treatments before their operations, the total number of patients with HCC included in this study was 123. The study was approved by the Institutional Review Board of the National Yang Ming University (IRB No. 1000041) and the user committee of the TLCN (No.070001 and No.120060). The clinicopathological profiles of the patients enrolled in the study are shown in Table I.

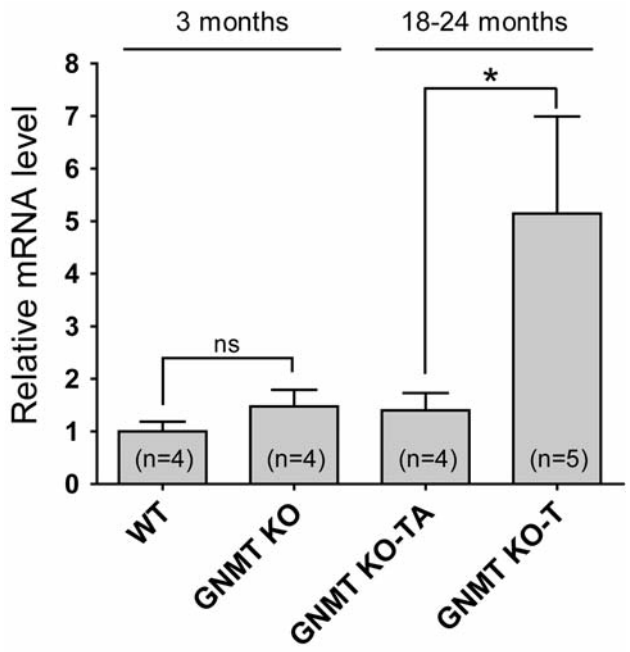


Figure 1. *Identification of FK506 binding protein 11 (Fkbp11) as a potential marker for HCC from glycine N-methyltransferase (Gnmt)$^{-/-}$ mouse model. The expression level of Fkbp11 was determined in liver tissues from wild-type (WT) and Gnmt$^{-/-}$ (GNMT KO) mice at age of 3 months; and in HCC (T) and tumor-adjacent (TA) liver tissue from Gnmt$^{-/-}$ mice at ages from 18-24 months. The expression level of each sample was normalized to the average of samples from wild-type mice. Fkbp11 was expressed at a level nearly 5-fold higher in HCC than in samples from other group. ns, non-significant; *p<0.05.*

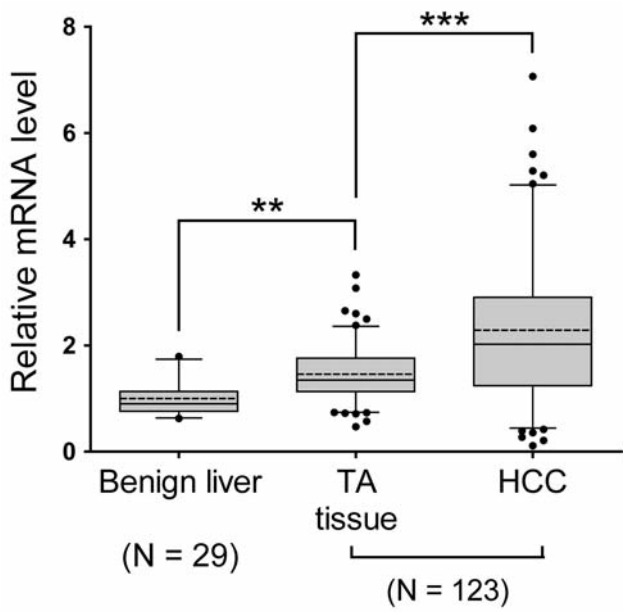


Figure 2. *The expression level of FK506 binding protein 11 (FKBP11) in human specimens. The expression level of each sample was normalized to the average of that for benign liver samples. A significantly and progressively elevated expression of FKBP11 from benign liver to tumor-adjacent (TA) tissue (1.5±0.5-fold higher) then to hepatocellular carcinoma (HCC) (2.3±1.4-fold higher) was observed. The full line represents the median and the dashed line represents the mean. The bottom and top of the box are the 25th and 75th percentiles. Whiskers (errors bars) above and below the box indicate the 95th and 5th percentiles. Dots represent outlying points. **p<0.01; ***p<0.001.*

*Mice*. *Gnmt*$^{-/-}$ mice with 129/B6 F2 background were generated as described previously (6, 19). An ultrasound machine with a 17-MHz probe (model HDI 5000, Philips, Seattle, WA, USA) was used to monitor liver tumor nodule formation in *Gnmt*$^{-/-}$ mice bi-weekly. All animal protocols were approved by the Institutional Animal Care and Use Committee of National Yang-Ming University (No. 1000518). Mice with nodules >0.5 cm were subjected to autopsy. Liver tissue samples were separated and divided into two parts: one was fixed in 3.7% formaldehyde for hematoxylin and eosin (H&E) staining; another was stored in liquid nitrogen for other analyses.

*Reverse transcription and real-time PCR*. Reverse transcription and real-time PCR were carried out as previously described (20). In brief, complementary DNA was produced from cellular RNA (1 μg) using a SuperScript II RNase H-Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA). Real-time PCR reactions were performed in 10-μl quantities of diluted cDNA sample, primers (100 nM), and a SYBR Green PCR Master Mix containing nucleotides, AmpliTaq Gold DNA polymerase, and optimized buffer components (Applied Biosystems, Foster City, CA, USA). Reactions were assayed using an Applied Biosystems Prism 7700 sequence detection system. The primers used for the real-time PCR were hFKBP11-F: 5' CTGGAGCAGAGTCTTCTCGACAT 3' and hFKBP11-R: 5' TCCATAGGCCAAGTGAGAAGGA 3' for human *FKBP11*; mFKBP11-F: 5' CTGGAGCAGAGTCTTCTCGACAT 3' and mFKBP11-R: 5' TCCATAGGCCAAGTGAGAAGGA 3' for mouse *Fkbp11*; and TATA box binding protein (TBP)-F: 5' CAGAAGTTGGGTTTTCCAGTCAA 3' and TBP-R: 5' ACATCACAGCTCCCCACCAT 3' for *TBP*. Predicted cycle threshold (CT) values were exported into EXCEL worksheets for analysis. Comparative CT methods were used to determine fold difference in gene expression relative to that for *TBP*.

*Statistical analyses*. Data are the mean±SD. Comparisons of mean values between groups were evaluated by two-tailed Student *t*-test or nonparametric Wilcoxon signed-rank test. A value of $p<0.05$ was considered to be statistically significant.

## Results

*Identification of FKBP11 as a novel HCC marker using the Gnmt*$^{-/-}$ *mouse model*. Previously, we used microarray analysis to evaluate the gene expression profiles in various pathways from early (11 weeks old), intermediate (dysplastic nodules) to late stage of HCC formation in both genders of *Gnmt*$^{-/-}$ mice (6). Fkbp11 was identified as a

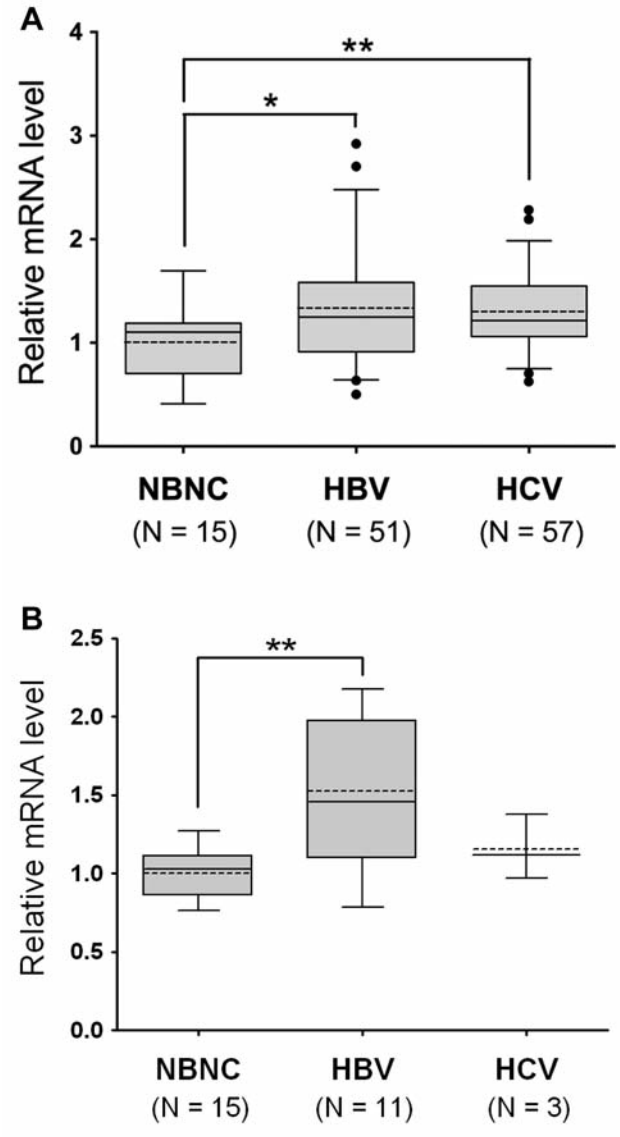


Figure 3. *Overexpression of FK506 binding protein 11 (FKBP11) is associated with viral hepatitis. The expression of FKBP11 was compared in tumor-adjacent (TA) tissues (panel A) and benign liver tissues (panel B) from patients with hepatitis B virus (HBV) or HCV or without viral hepatitis (NBNC). The expression level of each sample was normalized to the average of TA samples from the NBNC group. A significantly elevated level of FKBP11 in the TA tissue of both HBV and HCV groups was observed. The full line represents the median and the dashed line represents the mean. The bottom and top of the box are the 25th and 75th percentiles. Whiskers (errors bars) above and below the box indicate the 95th and 5th percentiles. Dots represent outlying points. *p<0.05; **p<0.01.*

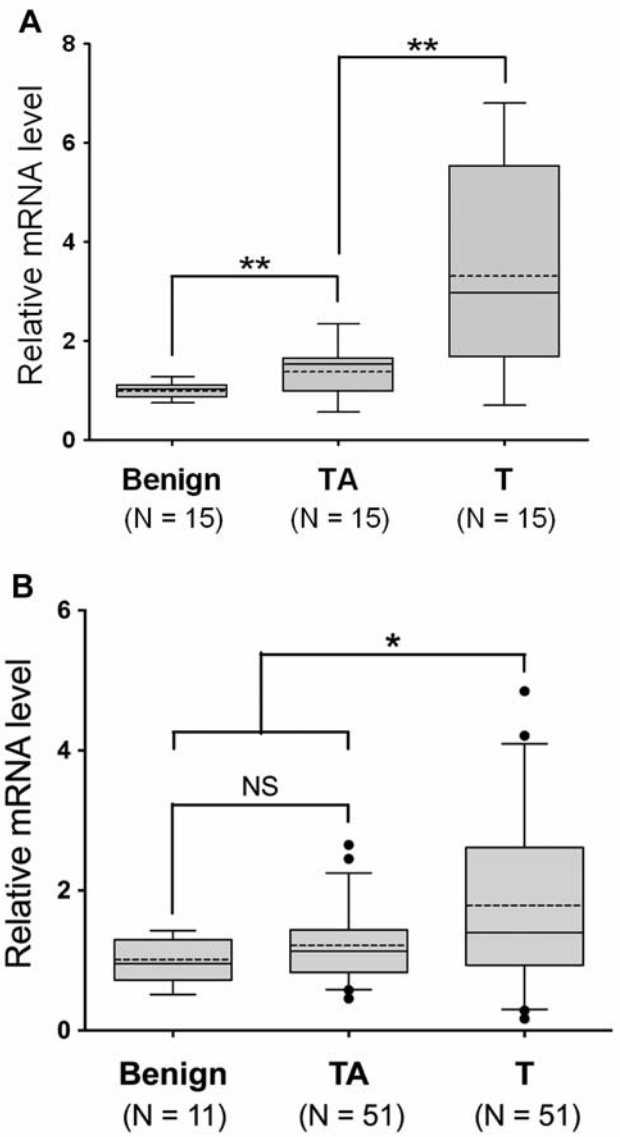


Figure 4. *Gradual elevation of FK506 binding protein 11 (FKBP11) expression during liver tumorigenesis. The expression of FKBP11 was compared among benign, tumor-adjacent (TA) tissue and hepatocellular carcinoma (HCC) tumor (T) tissues from the patients without viral hepatitis (NBNC) group (panel A) and HBV group (panel B). The expression level of each sample was normalized to the average of that for benign liver samples. A significantly and progressively elevated expression of FKBP11 from benign liver to tumor-adjacent tissue then to HCC was observed in the NBNC group. However, there was no significant difference in FKBP11 expression between the benign liver tissue and tumor-adjacent tissue in the HBV group. *p<0.05; **p<0.01.*

potential early HCC marker. We then verified the gene expression of *Fkbp11* in mouse specimens. As shown in Figure 1, *Fkbp11* gene expression was slightly higher in the liver tissue from *Gnmt*$^{-/-}$ mice than that from wild-type mice at the age of 3 months. Moreover, the level of *Fkbp11* was dramatically increased in the HCC tissues from *Gnmt*$^{-/-}$ mice ($p$<0.05). This suggests that the expression of Fkbp11 gradually increased as the disease progressed.

Table II. *Clinicopathological associations of FK506 binding protein 11 (FKBP11) mRNA expression level in patients with hepatocellulcar carcinoma.*

| Characteristic | | | *FKBP11* mRNA expression | | *p*-value |
|---|---|---|---|---|---|
| | | | Tumor tissue | Tumor adjacent tissue | |
| | | N | Mean±SD (range) | Mean±SD (range) | |
| Gender | Female | 40 | 4.98±3.37 (0.23-14.46) | 2.82±1.00 (0.96-5.11) | 0.001 |
| | Male | 83 | 4.54±2.57 (0.43-12.45) | 3.04±1.04 (1.48-6.81) | <0.001 |
| Viral infection | NBNC | 15 | 5.57±3.23 (1.18-11.46) | 2.33±0.72 (0.96-3.95) | 0.004 |
| | HBV | 51 | 4.57±3.01 (0.43-12.45 | 3.10±1.20 (1.17-6.81) | 0.005 |
| | HCV | 57 | 4.55±2.61 (0.23-14.46) | 3.02±0.86 (1.46-5.32) | <0.001 |
| Cirrhosis | No | 58 | 4.75±3.11 (0.43-14.46) | 3.02±1.20 (0.96-6.81) | 0.002 |
| | Yes | 65 | 4.62±2.63 (0.23-10.82) | 2.92±0.85 (1.17-5.32) | <0.001 |
| Stage* | Early | 60 | 4.52±2.74 (0.23-14.46) | 2.86±0.83 (0.96-4.57) | <0.001 |
| | Late | 63 | 4.84±2.96 (0.43-12.45) | 3.08±1.18 (1.17-6.81) | <0.001 |
| Tumor type | Solitary | 92 | 4.61±2.86 (0.23-14.46) | 2.99±1.03 (0.96-6.81) | <0.001 |
| | Multiple | 31 | 4.9±2.88 (0.43-10.82) | 2.91±1.02 (1.17-6.30) | 0.001 |
| Vascular invasion | Absent | 72 | 4.63±2.77 (0.23-14.46) | 2.82±0.80 (0.96-4.57) | <0.001 |
| | Venous invasion or vein tumor thrombosis | 51 | 4.76±2.99 (0.43-12.45) | 3.18±1.25 (1.46-6.81) | 0.003 |

HBV, Patients with hepatitis B virus surface antigen (HBs Ag); HCV, patients with antibody to HCV; NBNC, patients without HBs Ag and antibody to HCV. *Pathological: early stage, TNM stage = I; late stage, beyond TNM stage I (TNM stage=II+IIIA+IIIB+IIIC+IVA).

*Progressively elevated expression of FKBP11 during the development of HCC*. Next, the expression levels of FKBP11 in HCC specimens were analyzed and the results showed that its mean expression level in tumor tissue was significantly higher than that in tissue adjacent to the tumor (TA) ($p<0.001$; Figure 2). Importantly, we observed a statistically significant increase in FKBP11 level in TA tissues than in benign tissues which were collected from patients with hemangioma. These findings suggest a progressive elevation of expression of FKBP11 during the development of HCC.

Subsequently, clinicopathological data were correlated with the FKBP11 expression. As shown in Table II, the expression of FKBP11 was significantly higher in tumor tissue compared to TA tissue regardless of the gender, hepatitis status, HCC staging, cirrhosis status, tumor type and vascular invasion status of the patient (Table II). It is also important to note that an elevated level of FKBP11 can be found in early-stage HCC tumor tissues ($p<0.001$; Table II). Thus, FKBP11 has the potential to be an early marker for HCC.

*Overexpression of FKBP11 is associated with viral hepatitis*. Interestingly, a comparison of FKBP11 expression profile in TA tissues revealed an increase of *FKBP11* mRNA level in TA tissues from patients associated with viral hepatitis than in TA tissues from patient without hepatitis of viral etiology ($p<0.05$ for NBNC group *vs*. HBV group; $p<0.01$ for NBNC group *vs*. HCV group; Figure 3A). Moreover, this phenomenon was also observed in benign liver tissue (Figure 3B). The expression of FKBP11 was significantly higher in the benign liver tissues from patients with hemangioma who were positive for HBs Ag than in that from patients with hemangioma without viral hepatitis ($p<0.001$; Figure 3B). Due to the limited number of patients (n=3) with HCV infection, the comparison of FKBP11 expression between benign tissues of HCV group and NBNC group was not assessed.

Next, we analyzed the progressive expression of FKBP11 in the presence and absence of viral hepatitis. As shown in Figure 4A, the expression of FKBP11 gradually increased among benign liver tissues, TA tissues and HCC tumor tissues, with statistical significance. Despite FKBP11 beings highly expressed in HBV-associated tumorous tissue, however, there was no difference in the levels of FKBP11 between benign liver tissues and TA tissues in HBV-infected patients (Figure 4B). Together, these findings suggest that FKBP11 could be involved in the response to viral hepatitis and in the early stage of HCC development.

## Discussion

In this study, we identified FKBP11 as a novel marker for HCC. Overexpression of FKBP11 can be observed in tumor tissue of HCC regardless of the clinicopathological features. Importantly, we demonstrated that the level of FKBP11 increases progressively from benign liver tissue to HCC tumor tissue. Thus, we propose that FKBP11 has the potential to be an early marker for HCC.

It is noteworthy that we identified FKBP11 in the *Gnmt*$^{-/-}$ mouse model. GNMT has been reported to be a tumor suppressor for HCC (21). The expression of GNMT is down-regulated in more than 75% of patients with HCC regardless

of the disease etiology (22). Moreover, *Gnmt*$^{-/-}$ mice develop HCC spontaneously at high rates in both genders and the pathology of the liver tumors mimics multiple stages of human HCC development (6, 7). Therefore, we believe that the down-regulation of *GNMT* is an early event during HCC development and the *Gnmt*$^{-/-}$ mouse is a superior model for HCC-related studies. The present findings further support these notions.

To our knowledge, we are the first to demonstrate that FKBP11 expression is increased in the TA tissue of patients with NBNC and also in the benign liver tissue of patients with viral hepatitis in our study. These data indicated that *FKBP11* could be a gene responsive to some types of liver injury. It has been reported that FKBP11 is involved in protein folding (15). FKBP11 has been shown to be implicated in ER stress response in hepatocytes and beta cells (18, 23). Specialized secretory cells, such as hepatocytes, pancreatic β-cells, mature B-cells, and osteoblasts, where high-level secretory protein synthesis takes place, require a highly evolved mechanism to properly fold, process, and secrete proteins (24, 25). These collective mechanisms are termed the 'unfolded protein response' (UPR). The UPR is a normal physiological process required for organelle expansion to promote protein folding, secretion, calcium storage and lipid biosynthesis in the liver (24). However, when protein folding in the ER is disrupted by elevated secretory protein synthesis, overexpression and/or accumulation of mutant proteins, glucose deprivation, altered glycosylation, ER calcium depletion, shifting of redox status to a more reduced state, and overloading of cholesterol, create stress in the ER and lead to UPR activation (24, 25). It has been shown that metabolic disorders and abuse of alcohol or drugs can also induce ER stress and UPR (24). In addition, viral hepatitis, including infection with HBV and HCV, has been reported to be able to induce ER stress in hepatocytes (24, 26). More importantly, ER stress pathway (the UPR) has been reported to be involved in liver malignancy and HCC progression (27). Taken together, *FKBP11* might be an early response gene to liver injury events that indicate ER stress caused by metabolic disorders and hepatitis due to viral infection. Furthermore, as the disease progresses the level of FKBP11 increases dramatically.

In conclusion, we have shown in this study that the expression of FKBP11 increases progressively from benign liver tissue to HCC tumor tissue. This finding may have important implications regarding decision-making in diagnostic and therapeutic strategies for HCC. Further studies are needed to elucidate the underlying mechanisms and the functional consequences of FKBP11 overexpression.

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