The correlation of fibrinogen-like protein-1 expression with the progression and prognosis of hepatocellular carcinoma

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Research Article

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Abstract

Background

ConclusionsFibrinogen-like protein-1 (FGL1), as the member of FREP superfamily, had been widely concerned as a major immune inhibitory ligand of LAG-3. Although FGL1 expression levels didn't have significant difference in most of tumors via online data analysis, we found that it was down-regulated in liver cancer. Moreover, the correlation between FGL1 expression and the progression and prognosis of hepatocellular carcinoma (HCC) was still disputed.

Methods

In our study, firstly, we used bioinformatics analysis to define the expression profile and clinical significance of FGL1 in HCC. Then, we determined the FGL1 level in human HCC cell lines, tumor and normal liver tissues from HCC patients by western blot. Furthermore, tissue microassays were used to detect the expression of FGL1 through immunohistochemistry staining and verify whether FGL1 expression levels were associated with clinicopathological features of HCC patients.

Results

The results proved that FGL1 was down-regulated significantly in HCC cell lines and HCC tissues, corresponding with the results of our bioinformatics analysis. FGL1 expression levels in HCC were related to Edmondson grade and metastasis. Additionally, high FGL1 expression was related to better overall survival in HCC patients, indicating that the down-regulated FGL1 was correlated with poor prognosis and FGL1 might function as a tumor suppressor.

Conclusions

Taken together, expression levels of FGL1 may correlate with the progression and prognosis of HCC, and FGL1 could be a potential prognostic biomarker.

Introduction

According to the 2020 data on global cancer, hepatocellular carcinoma (HCC), one of the most frequent malignant tumors with 905,677 new cases and 830,180 deaths, ranks sixth among the most commonly diagnosed cancers, which also is the cancer with third highest mortality[1]. Due to the insidious onset of hepatocellular carcinoma and the limited availability of targeted drugs, the five-year survival rate of HCC patients in China is only 14.1%[2]. Reliable prognostic indicators may improve such poor condition. However, sensitivity and specificity of current biomarkers are quite disappointing[3, 4]. Therefore, it is critical to seek

effective biomarkers which can predict prognosis and guide treatment for HCC to further improve HCC clinical outcomes.

Fibrinogen-like protein-1 (FGL1), also considered to be hepassocin or hepatocyte-derived fibrinogen-related protein 1 (HFREP1), is a kind of liver-secreted protein with two disulfide-linked 34kd homodimers[5, 6]. Initially, FGL1 was reported to cloned in HCC, and found to be over-expressed in HCC[7, 8]. As the member of FREP superfamily, FGL1 was tightly correlated with the development and prognosis of a variety of diseases. Previous study had indicated that FGL1 conduced to activate mitosis and increased metabolic activity as well as closely related to obesity[9]. Under the normal physiological conditions, FGL1 could participate in fine-tuning systemic inflammation via contributing to form interaction of the liver and other peripheral tissues[10]. A study suggested that FGL1 promoted hepatocyte proliferation via stimulating the EGFR/ERK cascade via the Src-dependent pathway[11, 12]. In addition, FGL1 was considered as a liver regeneration factor with its increased expression during liver regeneration[13–15]. In summary, these results revealed that FGL1 had a vital effect on liver regeneration and protection. FGL1 expression not only affected hepatocyte regeneration but also regulated the growth and proliferation of tumor cells[16, 17]. Targeted disruption of FGL1 would accelerate the development of HCC, which hinted FGL1 as a therapeutic target for liver cancer patients[17]. Another study had shown that FGL1 was an important component of FGL1-LAG-3 pathways that promoted growth of tumors, implying that the double blockade of FGL1 and PD-1/PD-L1 might become an alternative option for these patients whose anti-PD therapy was not effective[18]. However, FGL1 was down-regulated in HCC, while upregulated in melanoma, lung, breast, and colorectal cancers compared with normal tissues[6]. Taken together, the overall action of FGL1 in HCC remained controversial.

In this study, we found that FGL1 showed lower level among most HCC cells and down-regulated in tissues of HCC patients. Further analysis using TMAs revealed that FGL1 expression was closely related to Edmondson grade and metastasis. Additionally, it had been proved that FGL1 was an independent factor for prognostic in patients with HCC through Cox regression analysis. Consistently, according to the Kaplan-Meier survival analysis, there was a significant difference between the expression of FGL1 and OS in HCC patients. Low FGL1 expression hinted a shorter prognosis. In conclusion, our results indicated that the FGL1 expression of HCC was significantly down-regulated and FGL1 might be used as a probable prognostic biomarker, serving for treatment choice and prognosis evaluation in patients with HCC.

Materials And Methods

Patient population and tissue samples

From January 1998 to December 2011, 238 HCC patients (patients age, 25-90 years, average, 57.48 years) in the Zhejiang Provincial People's Hospital were included in our study. Then, we conducted follow-up surveys to all patients for over 5 years from the time of surgical operation to December 2018 or death. To sum up, 238 HCC patients consisted of 47 (19.7%) women and 191 (80.3%) men in our study. At the initial diagnosis, 59% of the patients had tumors less than or equal to 5cm in diameter, while 41% with a tumor diameter greater than 5cm. A total of 65% of the patients suffered from Edmondson grade I/II and 35%

suffered from grade III. The number of patients with or without metastasis was 20 (8.7%) and 210 (91.3%), respectively. Our study was authorized by the Ethics Committee of the Zhejiang People's Hospital, and obtained informed consent from all patients.

Cell culture

HCCLM3, SKHEP1, 7721, SNU182, C3A, HEPAG2, HUH7, HEP3B, and LO2 cell lines were purchased from the National Collection of Authenticated Cell Cultures (NCACC, Shanghai, China). We cultured all cell lines in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) with 10% fetal bovine serum (Gibco, USA), 100 units/mL penicillin and 100 μg/mL streptomycin (Life Technologies, USA) under 5% CO2 at 37°C.

Western blot assay

Total proteins were extracted from the samples and HCC cell lines via using RIPA buffer with the protease inhibitor cocktail (Roche, Switzerland). A BCA kit (Beyotime, China) was applied to test the protein concentration. We boiled the proteins at 100°C for 5 minutes, then the protein extracts were separated by 12% SDS-PAGE, electrotransferred to 0.22μm PVDF membranes (Roche, Switzerland), and sealed with 5% skim milk for one hour. Next, the membranes were incubated with anti-GAPDH (Bioworld, USA, 1:2000 diluted) or anti-FGL1 (ab197357, Abcam, England,1:1000 diluted) primary antibody at 4°C overnight followed by washing in TBST for 3×10 minutes. After incubation with secondary antibody (1:2000 diluted) for one hour, we washed it in TBST for 3×10 minutes. FDbio-Dura enhanced chemiluminescence (ECL) reagent (FD8020, Fdbio science, China) was used to detect signals under the Alpha Innotech Fluor Chem-FC2 imaging system (ProteinSimple, USA). GAPDH was used as an internal control.

Bioinformatic analysis of FGL1 expression in HCC

We used the Gene Expression Profiling Interactive Analysis 2 (GEPIA 2) (http://gepia2.cancer-pku.cn/#index) online website to analyze FGL1 levels in tumors. First, we obtained the dot plot gene expression profile by performing a single gene analysis across multiple tumor samples and paired normal liver tissues. Next, we made use of expression DIY to analyze FGL1 expression in HCC with a box plot, setting the parameters as follows: gene; Gene A, FGL1; [Log_2FC] Cutoff, 1; p-value Cutoff, 0.01; Multiple Datasets; Datasets Selection (Cancer name), LIHC; Log Scale, Yes; Jitter Size, 0.4; Match Normal data, Match TCGA normal and GTEx data. Then we explored the possible relationship between FGL1 and clinical features by UALCAN online website (http://ualcan.path.uab.edu/). Finally, we performed survival analysis to plot a Kaplan-Meier curve of overall survival using the OncoLnc Database (http://www.oncolnc.org/).

Immunohistochemistry staining

Tissue specimens that included tissues of HCC and their adjacent normal liver tissues were fixed with formalin and embedded in paraffin. Firstly, we used xylene (Sinopharm, China) to perform the dewaxing of 5-μm paraffin-embedded TMA sections and rehydration in graded alcohols (Sinopharm, China). Tissue samples were incubated with 3% hydrogen peroxide (Sinopharm, China) in order to block endogenous peroxidases. Secondly, for the sake of the decrease in nonspecific protein binding, the sections needed to be incubated for 20 minutes by 1% bovine serum albumin (BSA;Sigma, Germany). Futhermore, the sections

were incubated with the anti-FGL1 polyclonal antibody (1:50; HuaBio, Hangzhou, China) at 25℃ for one hour, followed by a biotinylated secondary antibody (MXB, Fuzhou, China) at 37℃ for 30 minutes. Whereafter, we applied DAB chromogen (Gene Tech, Shanghai, China) to stain the TMA sections, counterstaining with Mayer's hematoxylin (HuaBio, Hangzhou, China). At last, we incubated all tissue sections with alcohol and xylene and used an inverted fluorescence microscope to observe the sections. According to the intensity and the proportion of positively stained cells, Immunohistochemical stainings of FGL1 were scored into four grades, judged independently by two pathologists.

Statistical methods

We used SPSS software 22.0 (Chicago, IL, USA) to analyze all data. The independence test of categorical variables used chi-square analysis or Fisher's exact test. In addition, Kaplan-Meier analysis was applied to evaluate any differences in survival. Variables with $P < 0.1$ in the univariate analysis were incorporated into the proportional hazard model of Cox for multivariable analyses. Differences could be deemed statistically significant at $P < 0.05$.

Results

Low FGL1 expression in HCC and down-regulation of FGL1 were connected with a poor prognosis of HCC patients

We first used GEPIA to determine FGL1 mRNA levels in multiple cancers in the database. Obviously, we discovered that FGL1 was decreased in LIHC tumor tissues compared with normal liver tissues (matched TCGA normal and GTEx data) (Fig. 1A, 1B). Further, we researched the possible relationship between FGL1 and clinical features on UALCAN online website. The results suggested that the FGL1 expression was significantly related to individual cancer stages, patients with higher cancer stage showed lower FGL1 expression (Fig. 1C). Based on the above results, we speculated that FGL1 might be correlated to the prognosis of HCC patients. In order to further explore the correlation between FGL1 expression and prognosis, we used OncoLnc database to conduct survival analysis on the expression status of FGL1. Our results demonstrated that FGL1 expression level was connected with the prognosis of HCC patients, and low FGL1 expression indicated poor OS (Fig. 1D).

Determination of FGL1 expression in human HCC cell lines and HCC tissues

According the positive results of online analysis, we verified the FGL1 expression in human HCC cell lines and HCC tissues. Firstly, the expression profile of FGL1 in human HCC cell lines was determined by western blotting. As you can see in Figure 2A, FGL1 showed lower expressions in LM3, SKHEP1, 7721, SNU182, C3A, HepaG2, HUH7, and Hep3B cells than in normal liver cell line (LO2). Secondly, we collected 11 pairs of fresh tissues from HCC patients to detect the expression profile of FGL1 in clinical samples. It had been revealed that the expression level of FGL1 was the lowest in HCC tissues than in normal tissues and peri-tumor tissues (Fig. 2B). Similarly, FGL1 showed lower expressions in HCC tissues than in normal liver tissues from

HCC patients (Fig. 2C, 2D). Moreover, IHC staining of TMAs indicated that FGL1 of HCC tissues had the lower expression level compared to that of adjacent normal liver tissues (Fig. 2E). In conclusion, above studies confirmed that FGL1 of HCC tissues was lower than that of normal tissues, corresponding with previous bioinformatic analysis.

Correlation analysis between FGL1 expression and clinicopathological parameters of HCC

On the basis of the median FGL1 expression level of tumors, we divided all patients into two groups. Next, we probed the associations between FGL1 expression levels and HCC's clinicopathological parameters. FGL1 expression levels in HCC were found to be related to Edmondson grade and metastasis (P <0.05). There was no significance between FGL1 expression and other clinical parameters (involving age, sex, tumor number, tumor sizes, microvascular invasion, hepatitis B surface antigen (HBS), cirrhosis, and AFP) (P >0.05, Table 1).

Table 1
Expression of FGL1 in hepatocellular carcinoma tissues

Clinical parameters	Number	FGL1 expression		χ^2	P value
		Low	High		
Age(years)				0.012	0.913
<55	90	13	77		
≥55	147	22	125		
Gender				0.894	0.344
Male	190	26	164		
Female	47	9	38		
Tumor size				0.651	0.420
≤50mm	138	18	120		
>50mm	95	16	79		
Tumor number				0.095	0.758
Single	194	28	166		
multiple	43	7	36		
Edmondson grade				4.069	0.044*
I+II	146	17	129		
III	78	17	61		
Metastasis				8.453	0.004*
M0	210	26	184		
M1	19	7	12		
Microvascular invasion				0.670	0.413
Absence	87	11	76		
Presence	88	15	73		
HBs antigen				0.015	0.904
Negative	46	7	39		
Positive	186	27	159		
Cirrhosis				3.049	0.081
Negative	78	16	62		

Clinical parameters	Number	FGL1 expression		χ^2	P value
		Low	High		
Positive	159	19	140		
AFP(μg/L)				0.297	0.586
<50	104	14	90		
≥50	86	14	72		

Prognostic significance of FGL1 expression for HCC

What's more, in order to analyze the prognostic factors of HCC, we had access to Cox regression to verify. Based on univariate Cox regression analysis, tumor number, Edmondson grade, metastasis, HBS antigen and FLG1 expression level might be prognostic factors for HCC (P <0.1). Multivariate Cox regression analysis was further applied to ensure that Edmondson grade, metastasis and FLG1 expression level were independent prognostic factors for HCC (P <0.05, Table 2). Consistently, it was proved that FGL1 expression correlated with OS in HCC patients on the Kaplan-Meier survival curve, with low FGL1 expression being related to a shorter OS (P<0.0001, Fig. 3).

Table 2. Univariate and Multivariate Cox regression of the clinicopathological parameters in HCC patients

Parameters	Univariate analysis				Multivariate analysis			
	Coefficient	HR	95.0%CI For HR	P	Coefficient	HR	95.0%CI For HR	P
Age（<55years/ ≥55 years）	-0.295	0.744	0.370-1.498	0.408				
Gender (Male/Female)	-0.199	0.819	0.370-1.814	0.623				
Tumor size (≤50mm/＞50mm)	0.376	1.457	0.659-3.218	0.352				
Tumor number (Single/multiple)	1.321	3.748	1.634-8.600	0.002*	0.519	1.680	0.941-2.998	0.079
Edmondson grade (I+II/III)	1.276	3.582	1.650-7.779	0.001*	1.076	2.932	1.751-4.909	0.000*
Metastasis (M0/M1)	0.842	2.320	0.894-6.023	0.084	1.402	4.062	2.139-7.712	0.000*
Microvascular invasion(-/+)	0.062	1.064	0.494-2.292	0.874				
HBs antigen (-/+)	-1.025	0.359	0.127-1.014	0.053	-0.033	0.968	0.532-1.760	0.915
Cirrhosis(-/+)	0.599	1.820	0.823-4.021	0.139				
AFP(＜50μg/L / ≥50μg/L)	0.251	1.285	0.598-2.762	0.520				
FGL1(-/+)	-0.924	0.397	0.171-0.922	0.032*	-0.861	0.423	0.247-0.725	0.002*

Note

" * " signifies P value<0.05.

Discussion

Although nowadays some progress has been made in the improvement of diagnosis and treatment for HCC in recent years, the prognosis of HCC patients is still poor. Even several markers such as AFP and AFU have been employed extensively, the specificity and sensitivity of these biomarkers are not sufficient[19]. It is false positives that make us hard to distinguish early-stage HCC from other liver disorders, for instance, acute hepatitis and cirrhosis[20]. Therefore, searching for more convenient, more reliable markers which can guide early diagnosis and indicate the prognosis of HCC is an important research direction.

FGL1 has been implicated as both a hepatic protectant and a hepatocyte mitogen conducive to mitogenic and metabolic activity[21]. In cases of liver injury or acute inflammation, FGL1 expression levels are also enhanced[10, 15, 22], and FGL1 can promote the proliferation of normal hepatocytes *in vivo*. Similarly, FGL1 also can affect the proliferation of HCC cells. Down-regulation of FGL1 in HCC cells may contribute to the growth and proliferation of HCC[23] It has been demonstrated that FGL1 promotes hepatic cell proliferation by an autocrine mechanism while inhibiting HCC cell proliferation via an intracrine pathway[24]. However, the exact role of FGL1 in HCC remains controversial. Therefore, we performed a series of experiments to verify.

Previous research had reported the down-regulation of FGL1 in HCC, and the expression level of FGL1 was strongly related to the differentiation statuses of tumors[25]. Consistently, we confirmed that FGL1 expression of HCC tissues had the lower expression level compared to normal liver tissues by GEPIA in our study. In addition, western blot analysis also confirmed that FGL1 showed lower expression in HCC cell lines (LM3, SKHEP1, 7721, SNU182, C3A, HepaG2, HUH7, and Hep3B cells) than in normal liver cells (LO2), and FGL1 of HCC tissues was lower than normal tissues and peri-tumor tissues. In order to further clarify its prognostic significance in liver cancer, we then performed survival analysis to plot a Kaplan-Meier curve of overall survival using the OncoLnc Database, showing that FGL1 expression level was connected with OS in HCC patients and down-regulated FGL1 was connected with poor OS (P<0.05). As we can see, FGL1 expression was significantly related to individual cancer stages in UALCAN online website. Our results of TMAs demonstrated that FGL1 expression levels in HCC were related to Edmondson grade and metastasis. Multivariate Cox regression analysis certified that FLG1 expression level could be regarded as a prognostic factor for HCC patients. As predicted, the Kaplan-Meier analysis demonstrated that down-regulation of FGL1 was related to poor prognosis of LIHC. Therefore, we hypothesized that the expression levels of FGL1 were linked to the progression and prognosis of HCC and that the prognosis of HCC might be improved by adjusting the expression of FGL1 in the future.

With consistent researches of cancers, FGL1 was paid more and more attention. There were other cancers whose prognoses were also associated with FGL1. Recent evidence showed that the upregulation of FGL1 was linked to poor prognosis of gastric cancer[16]. In LKB1 mutant lung adenocarcinoma, loss of FGL1 could promote angiogenesis and epithelial-mesenchymal transition[26]. Suppression of FGL1 contributed to inhibiting the expression of caspase 3 and PARP1, enhanceing gefitinib to perform the inhibitory and apoptosis-inducing actions in NSCLC cell line PC9/GR[27]. Yeonghoon Son et al. reported that sorafenib-

induced antitumous effects were relieved by knocking down FGL1[28]. FGL1 played an inhibitory role in the growth of HCC and acted a part as a tumor suppressor in the proliferation of HCC[23]. Above researches proved that FGL1 could regulate the growth and proliferation of tumor cells. Interestingly, high expression levels of FGL1 were related to high densities of $LAG3^{+}$cells, confirming that FGL1 was a high-affinity ligand for LAG-3[29]. The activation of T cell could be enhanced and anti-tumor immunity could be promoted by blocking the interaction of FGL1-LAG-3 pathway[18]. Hence, targeting the FGL1-LAG-3 pathway and anti-PD1 dual blockade might play a significant part in the treatment of HCC patients whose anti-PD1 therapy was not efficient. It had reported that FGL1 expression was decreased when oxysophocarpine down-regulated IL-6-mediated JAK2/STAT3 signaling, which resulted in enhancing the immunotherapy effect of $CD8^{+}$ T cells against HCC *in vivo* and *in vitro*[30]. Above evidences proved that FGL1 and LAG-3 were strongly connected with the clinicopathological features and prognosis of various tumors[31]. Our study was validated with clinical data on the basis of biogenic evidence and demonstrated its prognostic significance in HCC. Nevertheless, the underlying mechanisms of signaling pathways in HCC remained unclear. Further study and improvement were needed for further mechanisms, and we would continue to explore the detailed mechanisms between FGL1 and HCC in the future. In conclusion, our study indicated that FGL1 might be a potential prognosis indicator for HCC. And our study provided a basis for further study of FGL1 in HCC.

Conclusions

Expression levels of FGL1 may correlate with the progression and prognosis of HCC, and FGL1 could be a potential prognosis indicator in HCC.

Abbreviations

HCC
Hepatocellular carcinoma
FGL1
Fibrinogen-like protein-1
HFREP1
Hepatocyte-derived fibrinogen-related protein 1
FREP
Fibrinogen-related protein
LAG-3
Lymphocyte activation gene 3
TMA
Tissue microarray
SDS-PAGE
Sodium dodecyl sulfate polyacrylamide gel electrophoresis
PVDF
Polyvinylidene difluoride
GAPDH

Glyceraldehyde-3-phosphate dehydrogenase
TBST
Tris buffered saline with tween
ECL
Enhanced chemiluminescence
GEPIA
Gene Expression Profiling Interactive Analysis
LIHC
Liver hepatocellular carcinoma
IHC
Immunohistochemistry
TCGA
The Cancer Genome Atlas
OS
Overall survival
HBS
Hepatitis B surface antigen
NSCLC
Non-small cell lung cancer
TPM
Transcripts per million
LKB1
Liver kinase b1

Declarations

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article.

Competing interests

The authors declare that they have no competing interests.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

This study was authorized by the Ethics Committee of the Zhejiang People's Hospital, and obtained informed consent from all patients.

Consent for publication

Not applicable.

Authors' contributions

Nanni Hua, Hui Dong and Guoqing Ru collected and analyzed the data, performed the experiments, drew figures and tables, contributing in writing the manuscript. Shibing Wang, Xianglei He and Nanni Hua performed the statistical analysis and experiments; Xiangmin Tong received the funding for this study.Xiangmin Tong, Chen Yang, Shibing Wang participated in the design of the study, gave administrative or logistic support for this review, and reviewed drafts of the paper. All the authors agreed with the conclusions of this review and approved the final manuscript.

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Figures

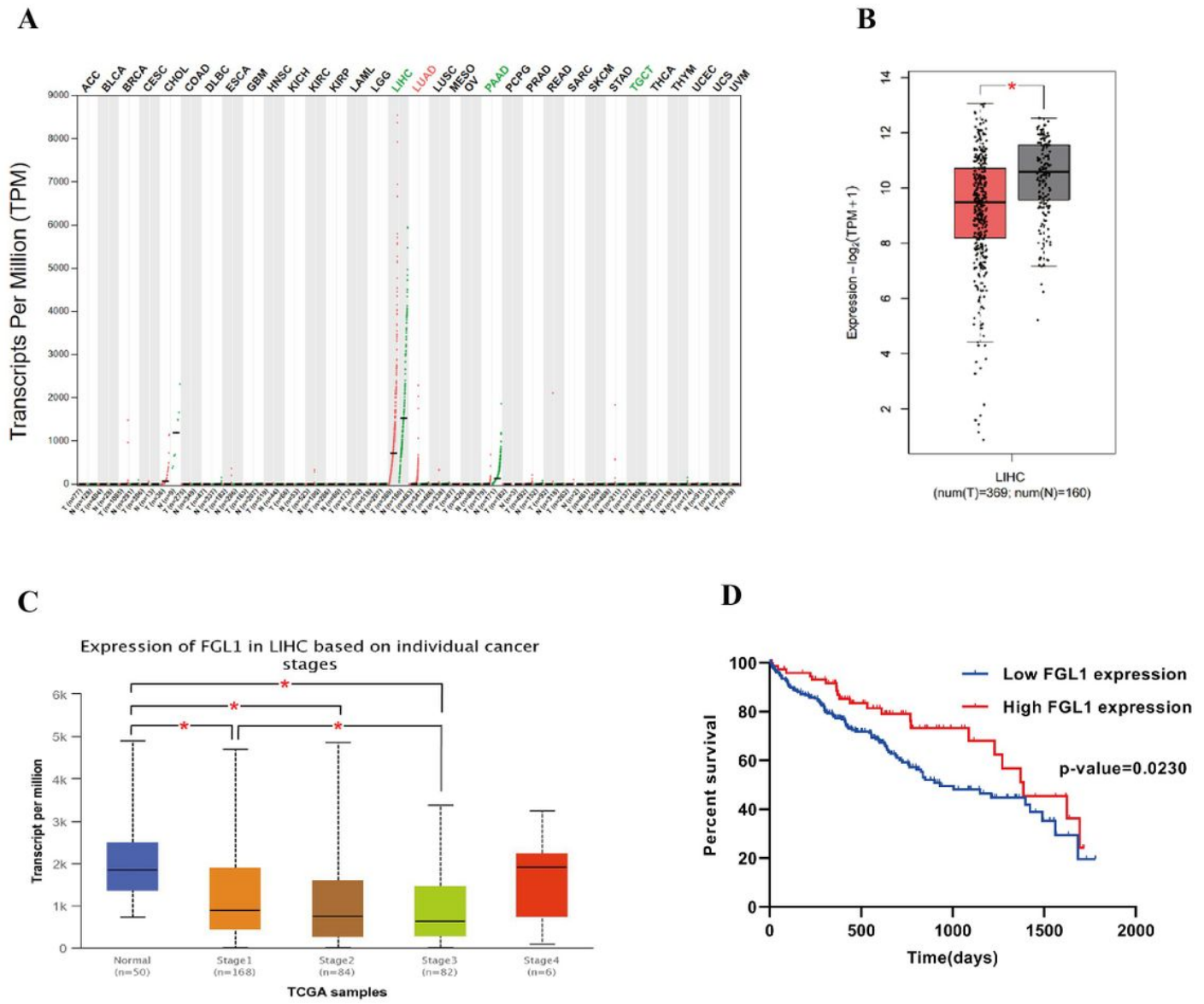

Figure 1

FGL1 expression of HCC tissues was down-regulated compared with paired normal tissues, and down-regulation of FGL1 was related to a poor prognosis of HCC. (A) FGL1 expression profile across all tumor samples and paired normal tissues on the basis of GEPIA. (B) The differential expression level of FGL1 in tumor and non-tumorous liver tissues. (C) FGL1 expression in LIHC based on individual pathological stages by UALCAN online website. (D) Kaplan-Meier survival curves of LIHC patients with low and high FGL1 expression based on the OncoLnc Database (P <0.05).

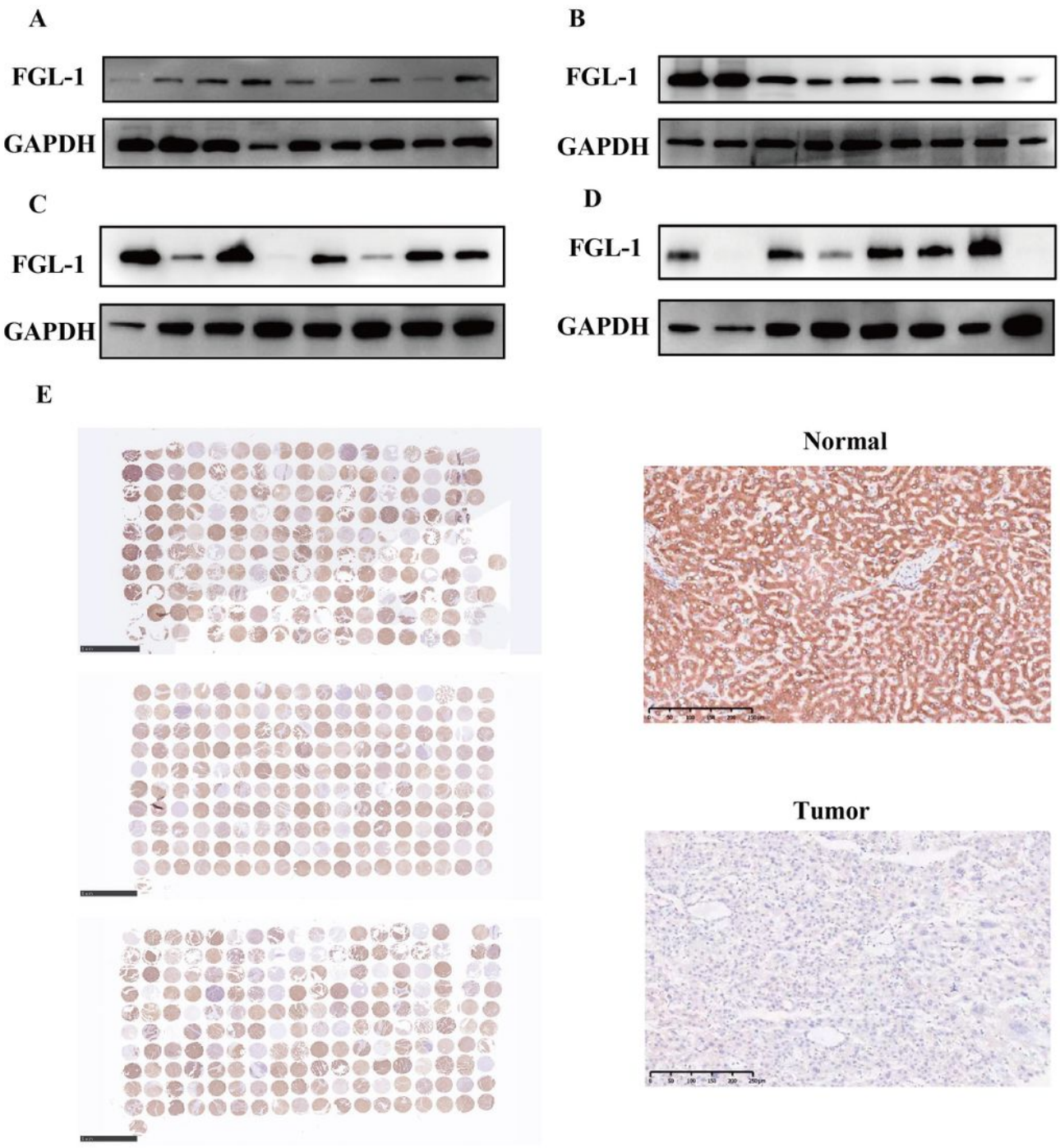

Figure 2

FGL1 expression showed obvious down-regulation in several human HCC cell lines and HCC tissues. (A) Determination of FGL1 expressions in several human HCC cell lines via western blot analysis. (B) Determination of FGL1 expressions with 3 pairs of HCC tissues and peri-tumor tissues and paired normal liver tissues via western blot analysis. (C-D) Determination of FGL1 expressions with 8 pairs of HCC tissues and paired normal liver tissues via western blot analysis. (E) IHC staining for tumor tissues and adjacent normal liver tissues from HCC patients in the TMA.

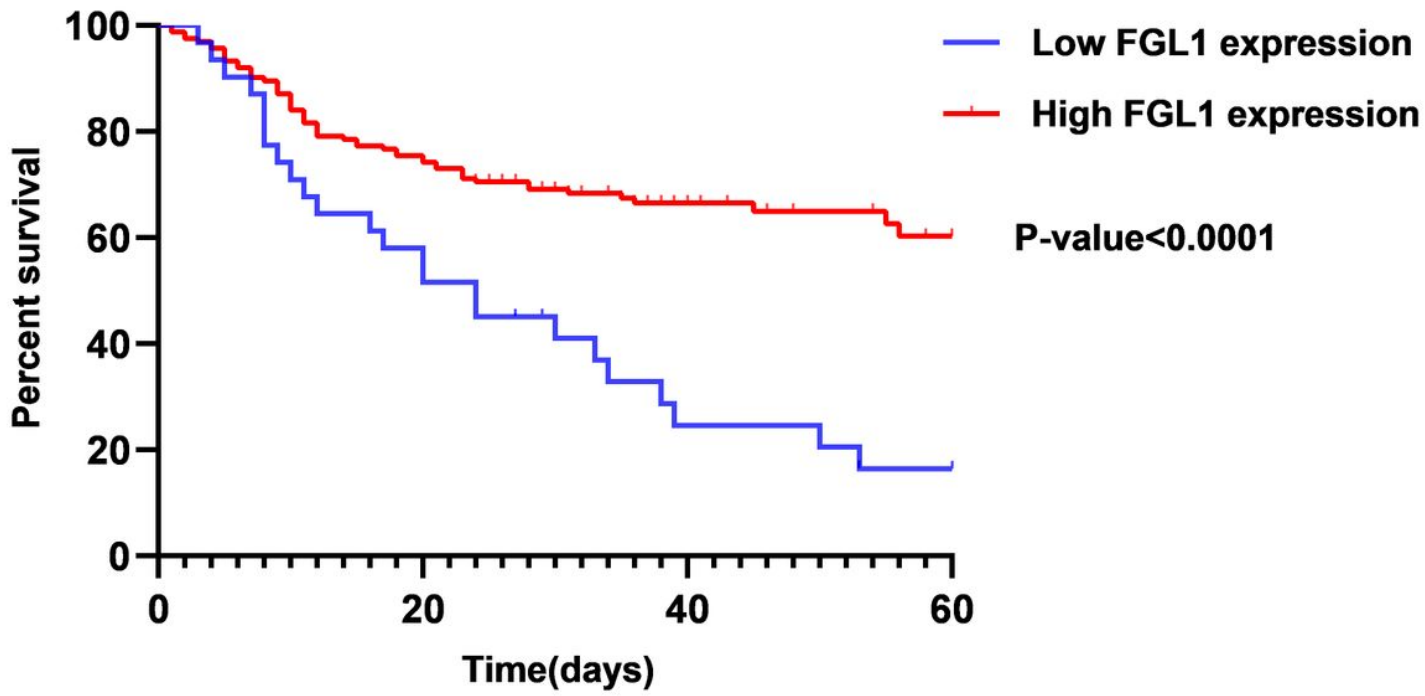

Figure 3

Kaplan-Meier survival analysis of HCC patients with different expression levels of FGL1 in the TMA.