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FKBP4 promotes glycolysis and hepatocellular carcinoma progression via p53/HK2 axis

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FKBP4, a member of the FK506-binding protein (FKBP) family, is a promising target for a variety of disorders, including cancer. However, its underlying molecular mechanism and potential function in hepatocellular carcinoma (HCC) are largely elusive. Therefore, we aimed to investigate the expression status, functional implications and underlying mechanisms of FKBP4 in HCC. Our bioinformatics analysis of TCGA LIHC datasets, ICGC LIRI-JP datasets and GEO datasets results showed FKBP4 was upregulated in HCC tissues. We also confirmed the elevated FKBP4 in clinical HCC samples. Additionally, quantitative RT-PCR results revealed FKBP4 was highly expressed in all five tested HCC cell lines. We also observed a correlation between elevated FKBP4 expression and poor prognosis in HCC patients. Loss of FKBP4 can inhibit the proliferation and migration in HCC cells. Furthermore, we found that silencing FKBP4 suppressed glucose uptake, lactic acid production and ^{18}F-FDG uptake compared with the control group. Mechanistically, our funding indicated that FKBP4 participates in glycolysis through p53 mediated HK2 signaling pathway, specially, FKBP4 knockdown promotes the expression and stability of p53 protein rather than affecting the transcription level. Finally, rescue experiments revealed that simultaneous knockdown of both FKBP4 and p53 partially reversed the inhibitory effects on HK2 protein levels and ^{18}F-FDG uptake. Our study elucidates a novel role of FKBP4 in promoting HCC development and glycolysis by modulating the p53/HK2 signaling pathway. Given the critical role of aerobic glycolysis in the progression of HCC, targeting FKBP4 may offer a new therapeutic strategy for treating this malignancy.

Hepatocellular carcinoma (HCC) is one of the most common cancers and the third leading cause of cancer-related death worldwide[1]. Hepatocarcinogenesis was a complicated process involving multiple factors that contribute to the initiation, development, and progression of HCC[2]. At present, the primary therapeutic agents for advanced HCC include multi-kinase inhibitors and immune checkpoint inhibitors. Unfortunately, the clinical benefit of oral inhibitors is limited[3]. Despite advancements in early diagnosis and specialized treatments for HCC, patients still suffer from poor prognosis and high recurrence rate, with median survival ranging from 6 to 20 months[4]. Due to liver dysfunction or metastasis, many patients lose surgical intervention opportunities. The molecular mechanism of HCC remains largely undefined, therefore, it is crucial to elucidate novel molecular mechanism and identify new therapeutic target for HCC.

FK506-binding proteins (FKBPs), intracellular receptors that binding FK506, belong to the immunophilin family and have been implicated in various biological pathways related to embryology, stress reaction, cardiac and neuronal function, indicating that they may play important roles in occurrence and development of different diseases including cancers. FKBP4, also known as FKBP52, is a high-molecular-weight immunophilins characterized C-terminal tetratricopeptide repeat (TPR) domains that can form complexes with the molecular chaperone Hsp90, and participate in protein-protein interactions. Evidence indicates that FKBP4 exerts relevant effects on various cancer cell types. For instance, in breast cancer models, especially in estrogen receptor (ER)-negative breast cancer, high expression of FKBP4 potentiate PI3K-Akt-mTOR signaling to facilitate tumor proliferation and survival[5]. Additionally, in Alzheimer's disease (AD) brain neurons, the abnormal FKBP4 decrease could hinder autophagy efficiency and contribute to the progression of the tauopathy by modulating microtubule associated protein tau (MAPT) secretion and accumulation during MAPT pathogenesis[6]. Notably, a recent study reported FKBP4 could integrate FKBP4/Hsp90/IKK with FKBP4/Hsp70/RelA complex, thereby potentiating the transcriptional activity and nuclear translocation of NF-κB, which in turn promotes the proliferation and metastasis of lung adenocarcinoma[7]. Upregulation of FKBP4, which has also been observed in HCC, was strongly related with disease staging and promoted the steroid hormone receptors translocating into

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the nucleus via the interaction with dynein[8]. However, the underlying molecular mechanism linking FKBP4 with metabolic process in HCC cells remains largely elusive.

Accumulating evidence has revealed that the energy production of cancer cells primarily depends on aerobic glycolysis rather than pyruvate oxidation[9]. Aerobic glycolysis plays an important role in the process of proliferation, growth, invasion, and treatment of many cancers, including HCC. The metabolic reactions of glycolysis are catalyzed by numerous enzymes. The changes of rate-limiting enzymes expression level, such as hexokinase (HK), phosphofructokinase (PFK), and pyruvate kinases (PKs) can greatly influence the progression and process of HCC. Among them, HK2 is the most efficient isoform and can efficiently catalyze the first important irreversible step by phosphorylating glucose, which is significantly highly expressed in HCC tissues and is closely related to the patient's clinical stages and poor prognosis[10,11]. Moreover, Dannielle DeWaal et al reported that knock-down of HK2 synergized with drugs like metformin or sorafenib in HCC patients and silencing hepatic HK2 suppressed tumor incidence in a hepatocarcinogenesis mouse model[12]. Thus, HK2 is considered to be a highly promising metabolic target for HCC treatment.

In this study, we characterized the biological role and regulatory mechanisms of overexpressed FKBP4 in HCC progression. Here we examined the expression status and investigated the potential functions of FKBP4 in HCC. Our bioinformatics analysis of The Cancer Genome Atlas (TCGA) LIHC datasets, International Cancer Genome Consortium (ICGC LIRI-JP) datasets and Gene Expression Omnibus (GEO) datasets and 29 pairs clinical samples results showed that FKBP4 was upregulated in HCC tissues. Additionally, quantitative RT-PCR results revealed that FKBP4 was highly expressed in 5 kinds of HCC cell lines, with elevated levels consistently correlating with poor prognosis in HCC patients. Our data demonstrated that loss of FKBP4 could inhibit proliferation and migration in HCC cells. Notably, we also discovered for the first time that FKBP4 participates in the glycolysis process through p53 mediated HK2 signaling pathway, suggesting that targeting FKBP4 might be a promising therapeutic strategy for treating HCC.

Results

FKBP4 is upregulated in multiple human cancers

To evaluate the importance of FKBP4, we analyzed the gene expression in both pan-cancer and healthy tissues sourced from the TCGA database. The results from the pan-cancer expression analysis demonstrated that higher levels of FKBP4 were present in tumor samples of CESC, LUAD, COAD, BRCA, ESCA, STES, STAD, PRAD, UCEC, LUSC, LIHC, READ, BLCA, HNSC and CHOL when compared with normal samples (Fig.S1). By contrast, we noted a significant down regulation of FKBP4 in tumor samples, such as GBM, GBMLGG, LGG, KIRP, KIPAN, THCA, KIRC, and KICH. By using the median as a cut-off point for analysis, Kaplan–Meier curves indicated that patients with higher FKBP4 levels exhibited shorter overall survival (OS) compared with those with lower FKBP4 levels in 9 types of tumor tissues. Among them, the expression level of FKB4 in tumor tissues consistent with prognostic survival analysis was only observed in LUAD and LIHC. As for disease-free interval (DFI), disease-specific survival (DSS) and progression-free interval (PFI) also demonstrated similar trends in LIHCs, where high FKBP4 expression was associated with poor prognosis (Fig.S2-S4). Consequently, we will further investigate the relationship and underlying mechanism of FKBP4 in HCC.

The high expression of FKBP4 in HCC is associated with cancer progression and prognosis

To explore the potential role of FKBP4 in HCC progression, we analyzed the RNA-seq data (FPKM values) obtained from TCGA database. The results showed that FKBP4 expression was significantly increased in HCC tissues ($n=364$) compared with normal tissues ($n=50$, $p<0.001$) (Fig. 1A, B). Survival analysis showed that FKBP4 expression level in tumor tissues was negatively associated with the overall survival time of patients with HCC (Fig. 1C). Consistently, we confirmed that the mRNA level of FKBP4 in tumor tissues was significantly higher than that in non-tumor tissues in ICGC RNA-seq dataset that enrolled 237 HCC patients (Fig. 1D, E). Survival analysis for this cohort also showed that patients with high FKBP4 expression consistently had poorer prognosis (Fig. 1F). In addition, the expression results of FKBP4 across GSE14520 and GSE64041 datasets mirrored those observed in both TCGA and ICGC datasets (Fig. 1G, H). To further evaluate the importance of FKBP4 in HCC, HPA database was used to compare the protein expression between normal and HCC tissues. As demonstrated in Fig. 1I, tissue microarray (HLivH060CS01) analysis further confirmed the increased expression of FKBP4 in HCC tissues.

FKBP4 expression correlates with patient clinicopathologic parameters

Furthermore, we evaluated the association between FKBP4 expression levels and patient clinical features in the TCGA database, revealing higher levels of FKBP4 expression to be evident in HCC patients with advanced clinical stage (Fig. 2B) and histologic grade (Fig. 2D). More specifically, the mRNA expressions of FKBP4 were higher in the groups of grade 2 (G2), grade 3 (G3), grade 4 (G4), stage II, III and IV compared with that in the control groups. In contrast, no significant association was observed between FKBP4 mRNA expression and age, gender (Fig. 2A, C). Subsequently, both univariate and multivariate regression analyses indicated that FKBP4 serves as an independent prognostic factor for OS, with hazard ratios (HR) of 1.476 (95% CI, 1.174-1.855) and 1.417 (95% CI, 1.110-1.809), respectively (Fig. 2E).

Functional analysis of FKBP4

To elucidate the biological functions and pathways of FKBP4 in HCC, we performed differential analysis between samples with high and low FKBP4 expression and regarded samples with low FKBP4 expression as controls (Fig. S5). We then used GO and KEGG enrichment assays to analyze the genes in each set. GO analysis showed

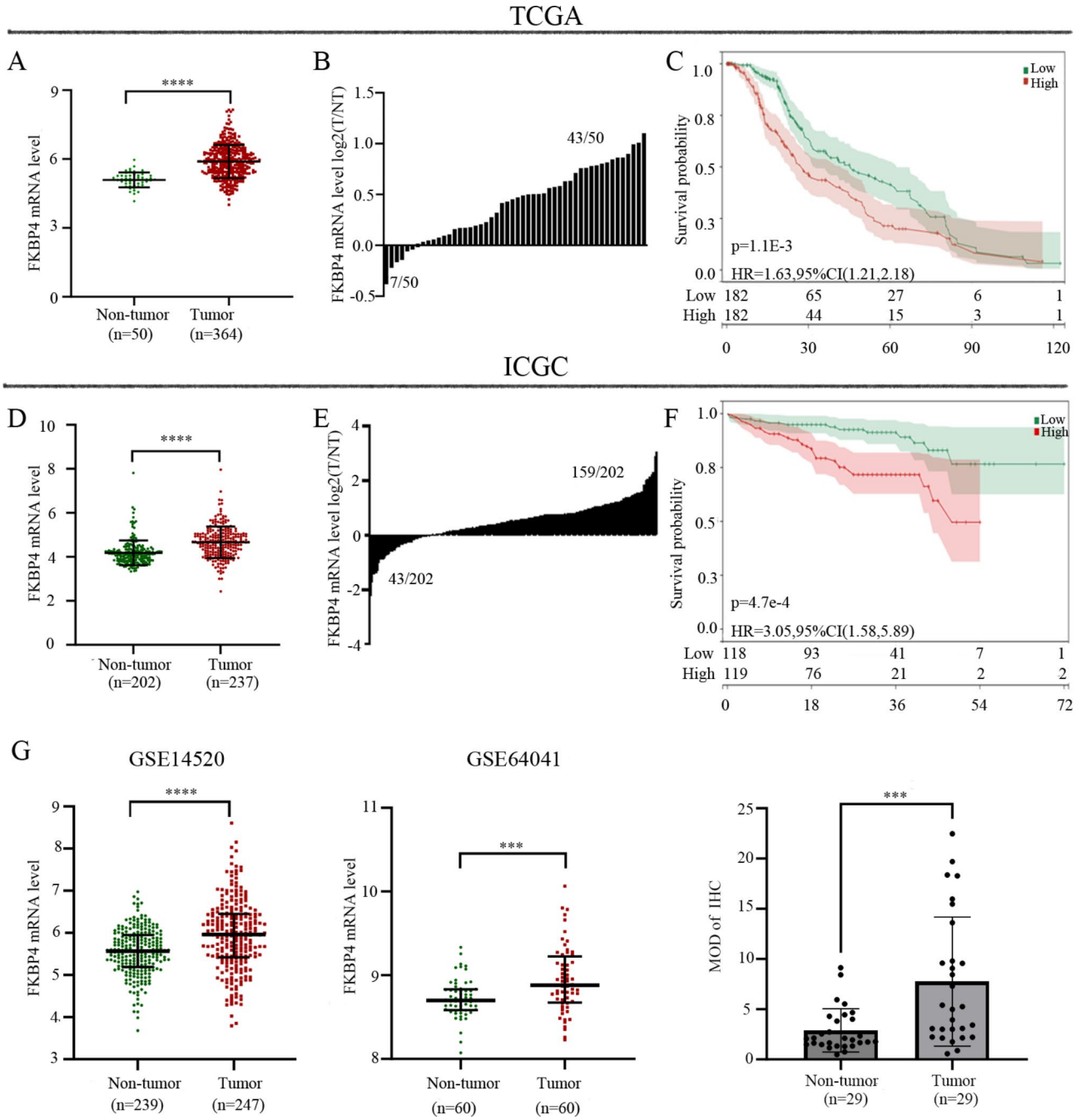
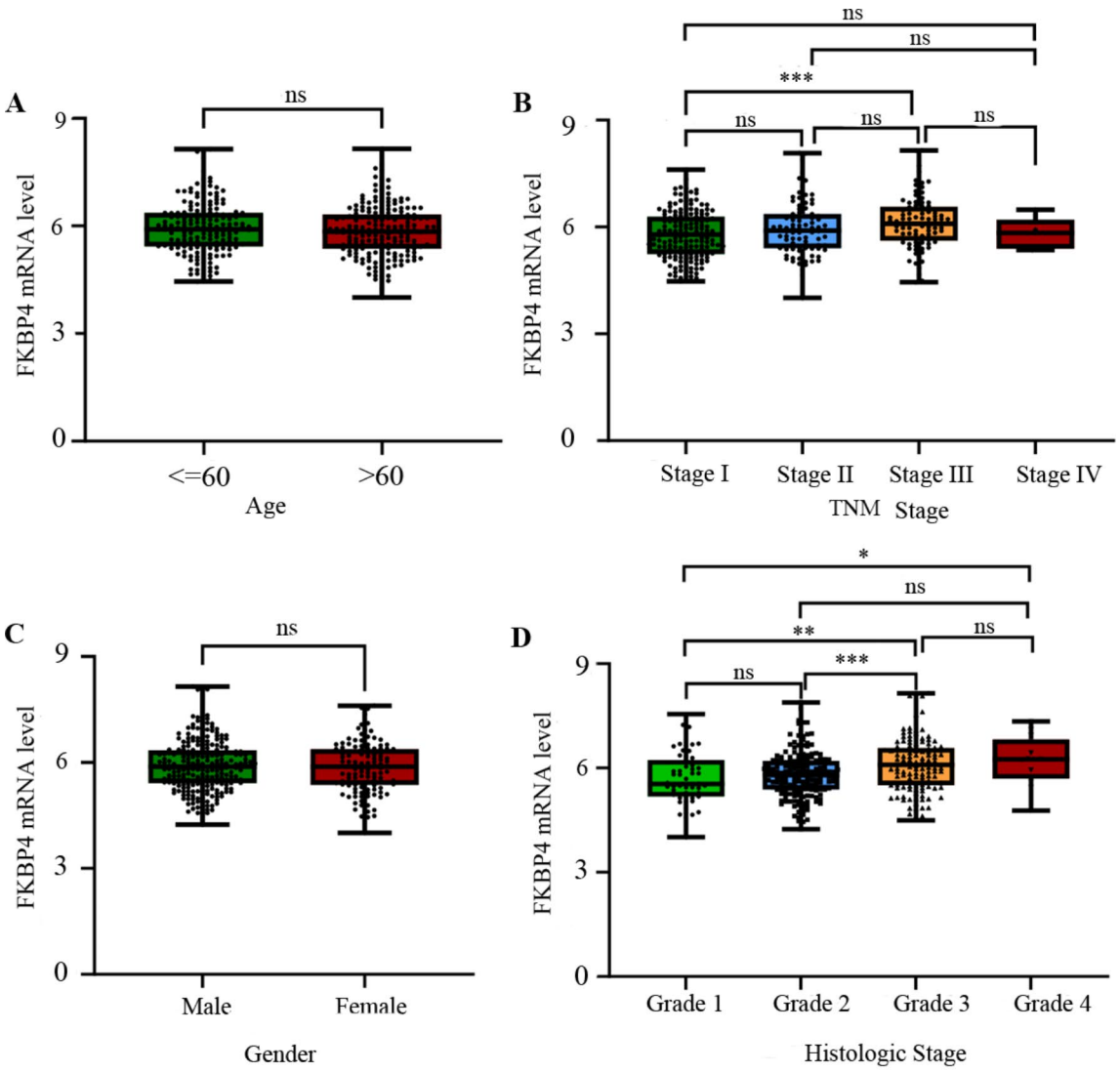


Fig. 1. Expression and predictive prognosis value of FKBP4 in patients with HCC. **A**, FKBP4 mRNA level in 364 HCC tissues and 50 non-tumor tissues from TCGA database. **B**, FKBP4 mRNA levels in HCC tissues and matched non-tumor tissues from the TCGA database ($n = 50$). T/NT indicates the expression level ratio of tumor tissues and matched non-tumor tissues. **C**, Kaplan-Meier analysis of FKBP4 mRNA and overall survival from 364 HCC tissues from TCGA database. **D**, FKBP4 mRNA levels in 237 HCC tissues and 202 non-tumor tissues from the ICGC database. **E**, Abnormal expression of *FKBP4* mRNA in 202 HCC tissues from the ICGC database. **F**, Kaplan-Meier analysis of *FKBP4* mRNA and overall survival from 237 HCC tissues from the ICGC database. **G**, Abnormal expression of FKBP4 mRNA in HCC tissues ($n = 247$) and non-tumor tissues ($n = 239$) from GSE14520. **H**, Abnormal expression of FKBP4 mRNA in HCC tissues and matched non-tumor tissues from GSE64041 ($n = 60$). **I**, IHC quantitative analysis of FKBP4 protein expression in 29 pairs of human hepatocellular carcinoma tissues and corresponding adjacent liver tissues. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.



E

	Univariate analysis		Multivariate analysis	
Variable	HR (95%CI)	*p* value	HR (95%CI)	*p* value
Age	1.249(0.881-1.771)	0.209		
Gender	1.228(0.861-1.749)	0.257		
TNM stage	2.448(1.689-3.548)	<0.0001	2.385(1.639-3.471)	0.0001
Grade	1.12(0.781-1.606)	0.539		
FKBP4	1.476(1.174-1.855)	0.001	1.417(1.110-1.809)	0.005

Fig. 2. Association of FKBP4 expression with clinicopathologic parameters based on TCGA database. The correlation between FKBP4 mRNA expression and clinical features, including age (**A**), TNM Stage (**B**), gender (**C**), histologic stage (**D**). Univariate and multivariate cox regression analyses identified FKBP4 as an independent clinical predictor for OS (E). $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; $^{****}p<0.0001$.

that the abnormal increase in FKBP4 expression was related to small molecule metabolic process, organic acid metabolic process, catalytic activity, oxidoreductase activity, extracellular region part, extracellular space, and closely related to other functions (Fig. 3A). In terms of the pathway enrichment, the DEGs from the TCGA cohort were obviously enriched in metabolism-related pathways, glycolysis and gluconeogenesis (Fig. 3B).

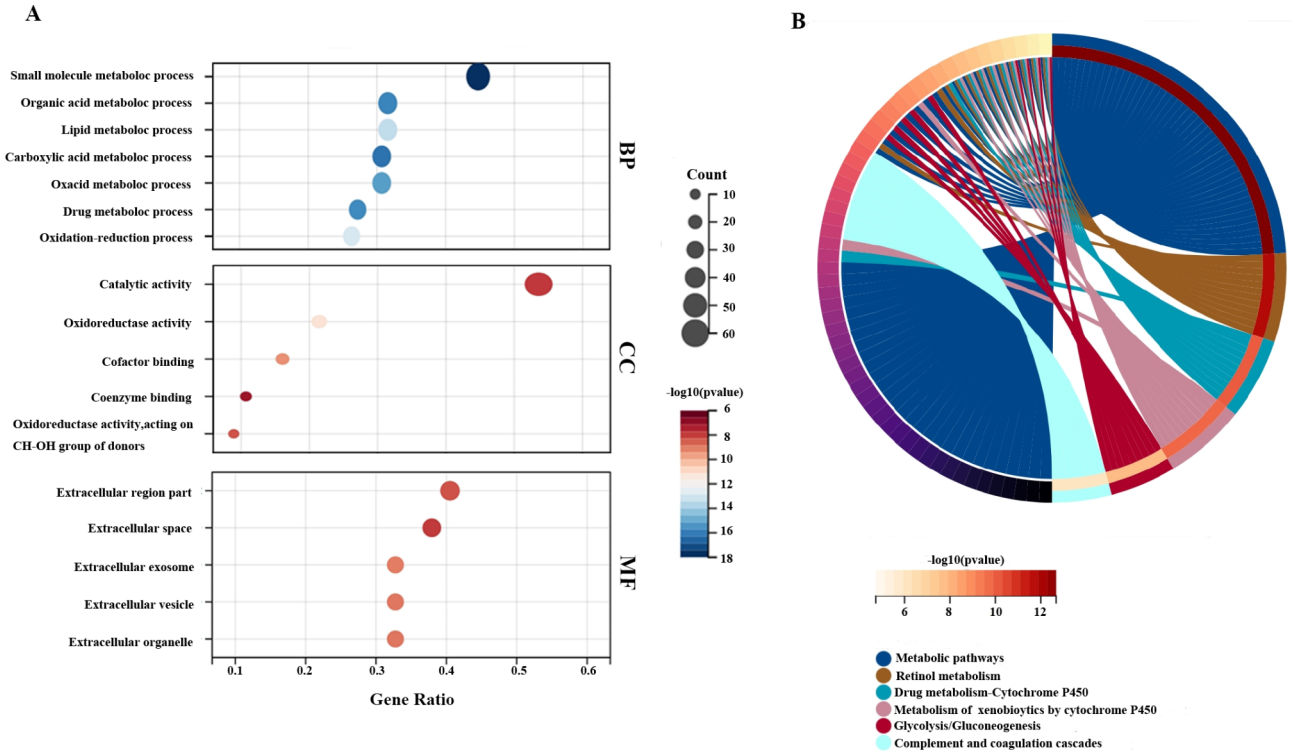


Fig. 3. Functional analysis of FKBP4. KEGG (**A**) and GO (**B**) enrichment analysis of differential genes of FKBP4 (|LogFC| > 1, adjusted p value < 0.05).

FKBP4 knockdown impairs cell growth and proliferation

To explore the FKBP4 expression status in HCC cells, qRT-PCR and western blot were conducted to detect FKBP4 mRNA and protein level in five HCC cell lines as well as normal liver cell line LO2. We found that FKBP4 expression was significantly increased in all tested HCC cells compared with LO2 (Fig. 4A, B and C), suggesting a potential role for FKBP4 in promoting HCC progression. To further explore this hypothesis, FKBP4 expression was knocked down in both SMMC7721 and Huh1 cells using siRNA targeting FKBP4 (siFKBP4#2), which exhibited more than 80% reduction efficiency of FKBP4 mRNA level (Fig. 4D and E). CCK-8 assay showed that FKBP4 knockdown markedly decreased the viability of SMMC7721 and Huh1 cells at 48-, 72- or 96-hours post-treatment (Fig. 4F and G). In addition, interference of FKBP4 suppressed the migration capability of both SMMC7721 and Huh1 cells (Fig. 4H and I). Moreover, FKBP4 downregulation obviously suppressed colony formation ability in both HCC cell lines (Fig. 4J and K). Collectively, these results suggest that silencing FKBP4 could repress HCC progression in vitro by reducing cell viability, colony formation, and proliferation.

FKBP4 down-regulation suppresses glycolysis by decreasing HK2 in HCC cells

Glucose metabolism is increasingly recognized as a promising therapeutic target in cancer treatment paradigms. To explore whether FKBP4 influences cellular metabolism in HCC, we analyzed a series of glycolytic related enzymes based on the TCGA LIHC data sets. As shown in Fig. 5A, glycolytic enzymes such as ENO1, G6PD, HK2, LDHA, PGK1, PKM, were found obviously increased in FKBP4 high expression group.

Then, we examined the glucose uptake and lactate production status in HCC cells. As anticipated, FKBP4 silencing reduced glucose uptake and lactate production in Huh-1 and SMMC7721 cells (Fig. 5B and C). ^{18}F-FDG is the most common clinical PET imaging agent in clinical practice currently, and is widely used in tumor diagnosis and identification based on the glycolysis of tumor cells. Therefore, we also investigate the effect of FKBP4 expression on glycolysis by using ^{18}F-FDG uptake experiment in Huh-1 and SMMC7721 cells. Consistently, the results showed that knockdown of FKBP4 significantly decreased the uptake rate of ^{18}F-FDG (Fig. 5D), reinforcing that knockdown of FKBP4 inhibited glycolysis activity in vitro.

In order to further elucidate the molecular mechanism of FKBP4 effect on glucose metabolism in HCC cells, we performed western blot to check the changes of glycolysis related molecules after FKBP4 silencing. Our data showed that knockdown of FKBP4 significantly impaired HK2 expression level in both Huh-1 and SMMC7721 cells compared with control group (Fig. 5E and F).

FKBP4 knockdown promotes the expression and protein stability of p53 in HCC cells

The reprogramming of glucose metabolism in tumor cells has been attributed to various causal factors. Among them, p53 has been considered as a major glucose metabolic regulator via TP53-induced glycolysis and apoptosis regulator (TIGAR), phosphoglycerate mutase (PGAM). Here, we detected p53 expression after knocking down

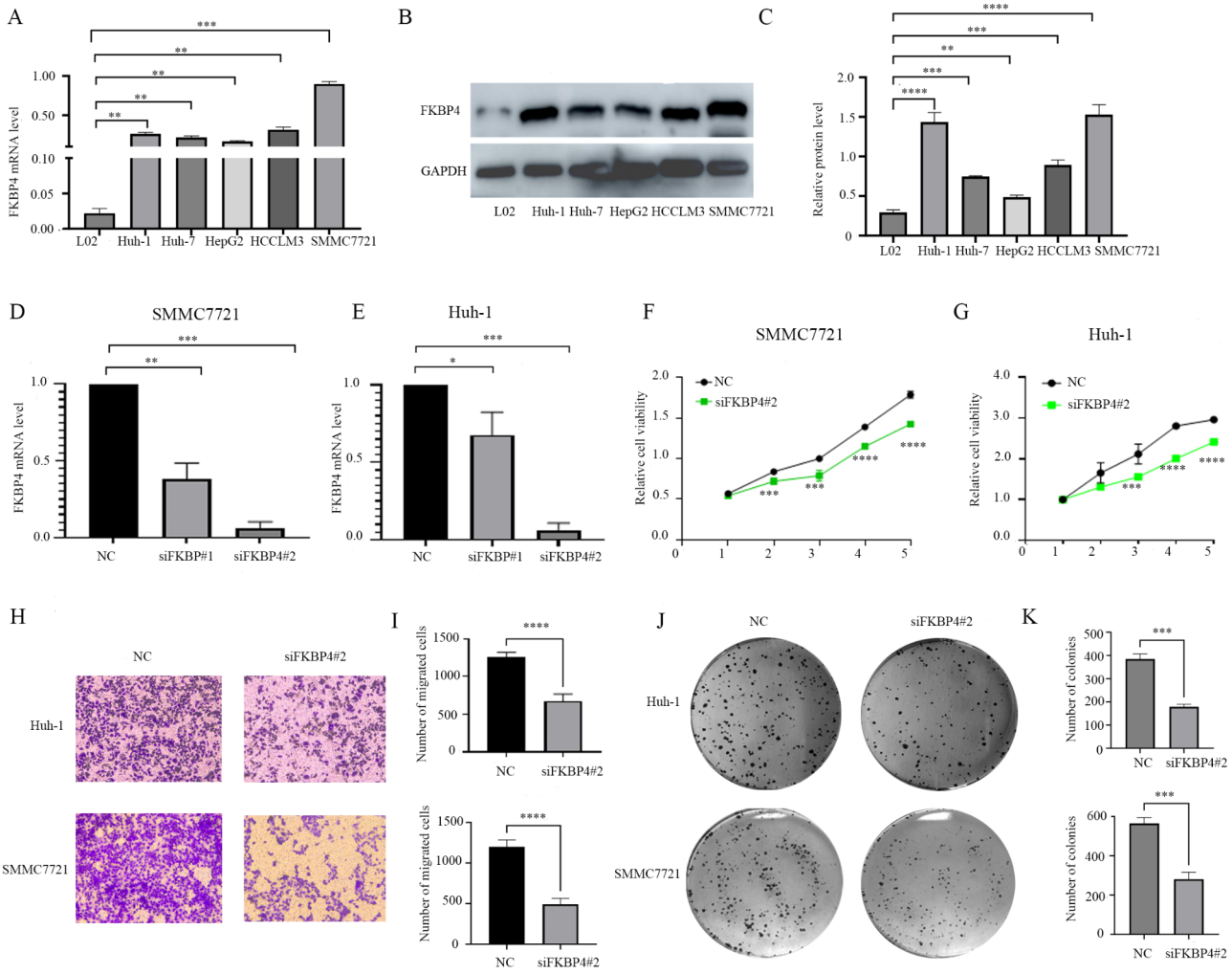


Fig. 4. FKBP4 knockdown impairs cell growth and proliferation. **A**, **B** and **C**, the mRNA and protein levels of FKBP4 were higher in HCC cell lines than in normal liver cell; **D** and **E**, knockdown efficiency of FKBP4was measured by qRT-PCR in SMMC7721 and Huh-1 cells. **F** and **G**, FKBP4 knockdown on cell proliferation rate were detected by CCK8 assay in SMMC7721 and Huh-1 cells. **H** and **I**, Effects of FKBP4 knockdown on metastasis were detected by Transwell assays in SMMC7721 and Huh-1 cells. **J** and **K**, the effects of FKBP4 knockdown on cell proliferation rate were detected colony-formation assay in SMMC7721 and Huh-1 cells. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

FKBP4 in HCC cells. We observed that depletion of FKBP4 increased p53 protein level (Fig. 6A and B). We then used qRT-PCR to detect the p53 mRNA level in both Huh-1 and SMMC7721 cells in which FKBP4 was knocked down. However, the p53 mRNA level remained unchanged in both cell types (Fig. 6C). We next used the turnover assay to test whether FKBP4 could affect the degradation of p53 protein. As shown in Fig. 6D and E, knockdown of FKBP4 significantly extended the half-life of endogenous p53 protein in both Huh-1 and SMMC7721 cells. The above results suggest that knockdown of FKBP4 upregulates p53 by inhibiting its degradation, rather than promoting its transcription. The function of p53 is predominately regulated at the post-translational levels, such as phosphorylation, acetylation, and methylation for its protein stability. Consistently, the elevation of p53 protein levels in FKBP4-knockdown cells was accompanied by an increase in p53-S15 phosphorylation level, rather than p53-S37 phosphorylation level (Fig. 6F and G). Therefore, we hypothesized that FKBP4 knockdown in HCC cells is a novel regulator of p53 stability and is accompanied by an increase in p53-S15 phosphorylation level.

P53 is a major mediator of FKBP4-induced HK2 activation

The above findings led us to further investigate whether FKBP4 influences glycolysis by p53-HK2 axis. Indeed, we found that the decrease of HK2 protein level could be partially rescued by treatment with p53 siRNA#1, p53 siRNA#2 in both Huh-1 and SMMC7721 cells (Fig. 7A and B). To elucidate the effects of FKBP4 and p53 on the glycolysis process, we carried out the ^{18}F-FDG uptake experiment (Fig. 7C and D), and as expected, we observed that the loss of FKBP4 accompanied by p53 knockdown can also partially raise the uptake rate of ^{18}F-FDG. Therefore, we revealed that FKBP4 promotes glycolysis of HCC via modulating the p53/HK2 signal pathway.

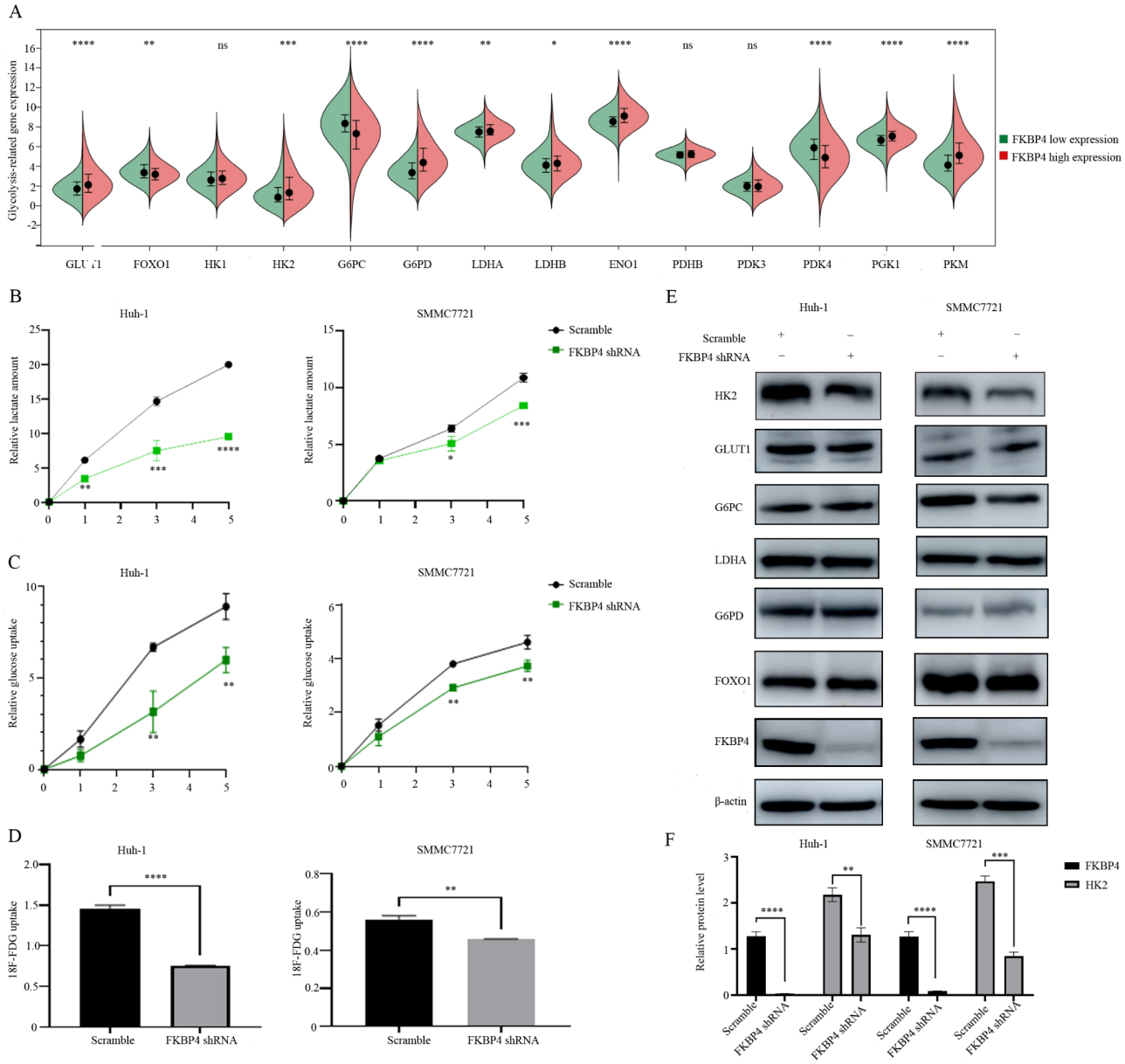


Fig. 5. FKBP4 down-regulation suppresses glycolysis by decreasing HK2 in HCC cells. **A**, glycolytic related enzymes were analyzed in TCGA LIHC datasets. Comparison of the control groups and FKBP4 stable knockdown groups in response to lactate production (**B**), glucose uptake (**C**) and ^{18}F-FDG uptake (**D**) in both Huh-1 and SMMC7721 cells. **E** and **F**, western blot was applied to detect the protein level of glycolytic related enzymes in both SMMC7721 and Huh-1 cells. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

Discussion

HCC is one of the most lethal malignant digestive system cancers worldwide with high morbidity and mortality. Although surgical resection is generally an effective treatment option for patients with early-stage HCC, the 5-year survival rate for patients with more advanced disease is less than 12.5%[13]. More intensifying studies are aimed at elucidating the detailed mechanisms of HCC development. Dysregulation of co-chaperone protein FKBP4 has been proposed to have a highly expression pattern in HCC serum. However, the function and mechanism of FKBP4 in HCC development and progression remain unclear. In this study, we demonstrated that the expression of FKBP4 was significantly upregulated in HCC tissues, associated with various clinicopathological characteristics and poor prognosis among HCC patients. Subsequent bioinformatic analysis and functional experiments revealed that FKBP4 plays a critical role in glycolysis process. Furthermore, we demonstrated that FKBP4 influences glycolytic process through p53 mediated HK2 signal pathway. Therefore, we concluded that FKBP4 may play an important role in promoting HCC progression.

As a member of FK506-binding proteins (FKBPs) family, FKBP4 has been proposed to have diverse functions in varieties of human diseases associated with hormone-dependent, stress-related, and neurodegeneration[14–16].

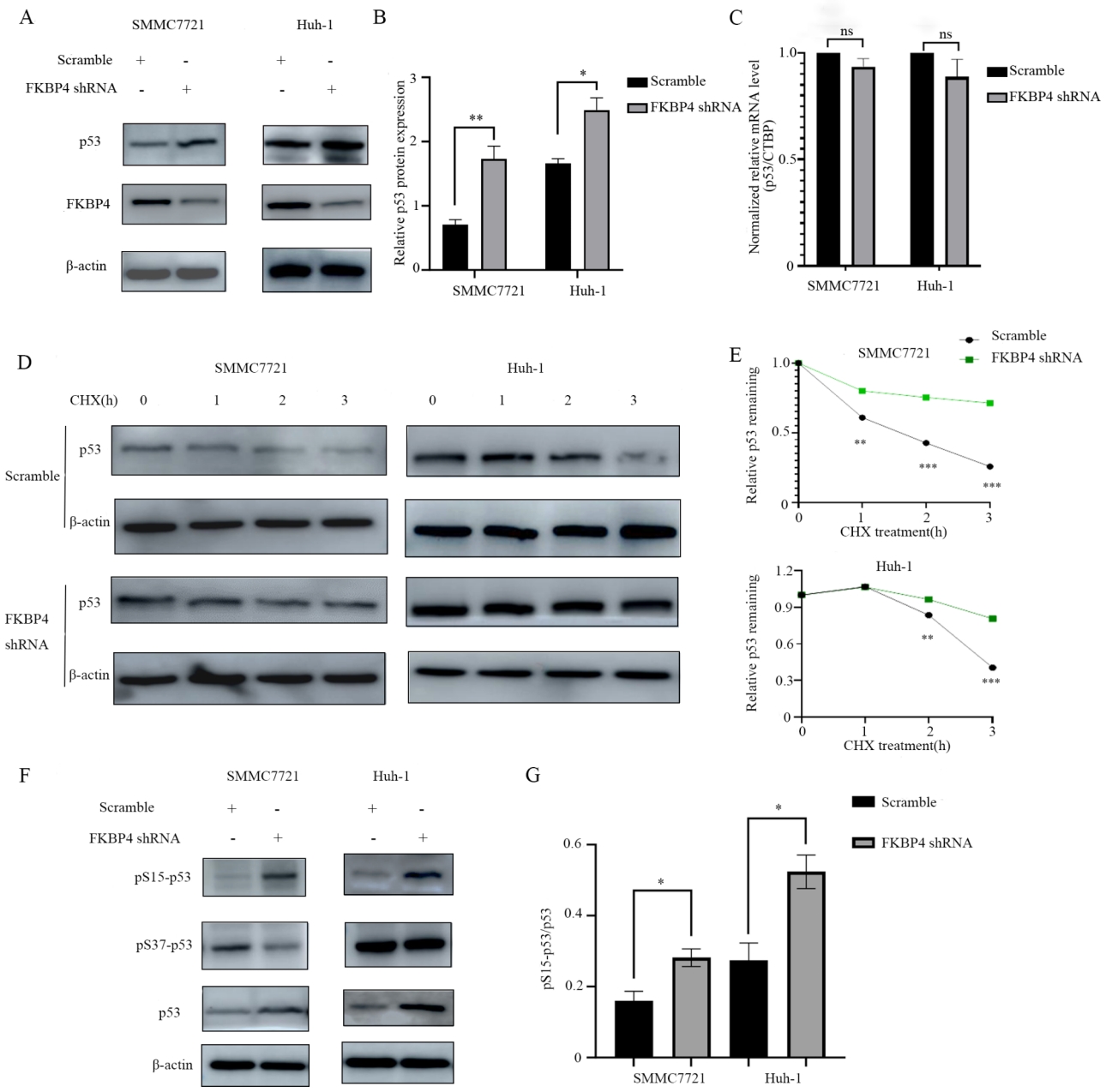


Fig. 6. FKBP4 affects phosphorylation of p53 at Ser15, and activation of the p53 signaling pathway. **A** and **B**, western blot analysis of p53 in Huh-1 and SMMC7721 cells between the control groups and FKBP4 stable knockdown groups. β-actin was used as the internal control. **C**, The mRNA levels of p53 were measured by qPCR (SYBR Green) in Huh-1 and SMMC7721 cells between the control groups and FKBP4 stable knockdown groups. CTBP was used as the internal control. Data shown are the mean ± s.d. Turnover assay of p53 protein in Huh-1 cells (**D**) and SMMC7721 cells (**E**) that had been transfected with FKBP4 shRNA or control expression vector with cycloheximide (CHX) (100 μg/ml) treatment at 48 hours post transfection for the indicated times. **F**, western blot analysis of the phosphorylation of p53 protein level in Huh-1 and SMMC7721 cells between the control groups and FKBP4 stable knockdown groups. β-actin was used as the internal control. **G**, Quantification of western blot presented in F was performed for pS15-p53, pS37-p53. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

Our study also found that FKBP4 expression was significantly upregulated in most cancer types including breast cancer, osteosarcoma, and squamous lung carcinoma, potentially contributing to tumorigenesis. However, its abundance and biological activity in the development and progression of HCC are still obscure. Liu YS. et al have reported that FKBP4 expression was increased in HCC tumor tissues[8]. Consistent with this study, our result also demonstrated that the FKBP4 expression level in HCC tumor tissues was significantly higher than that in the non-tumor tissues in TCGA database cohorts as well as ICGC database, GEO datasets and clinical

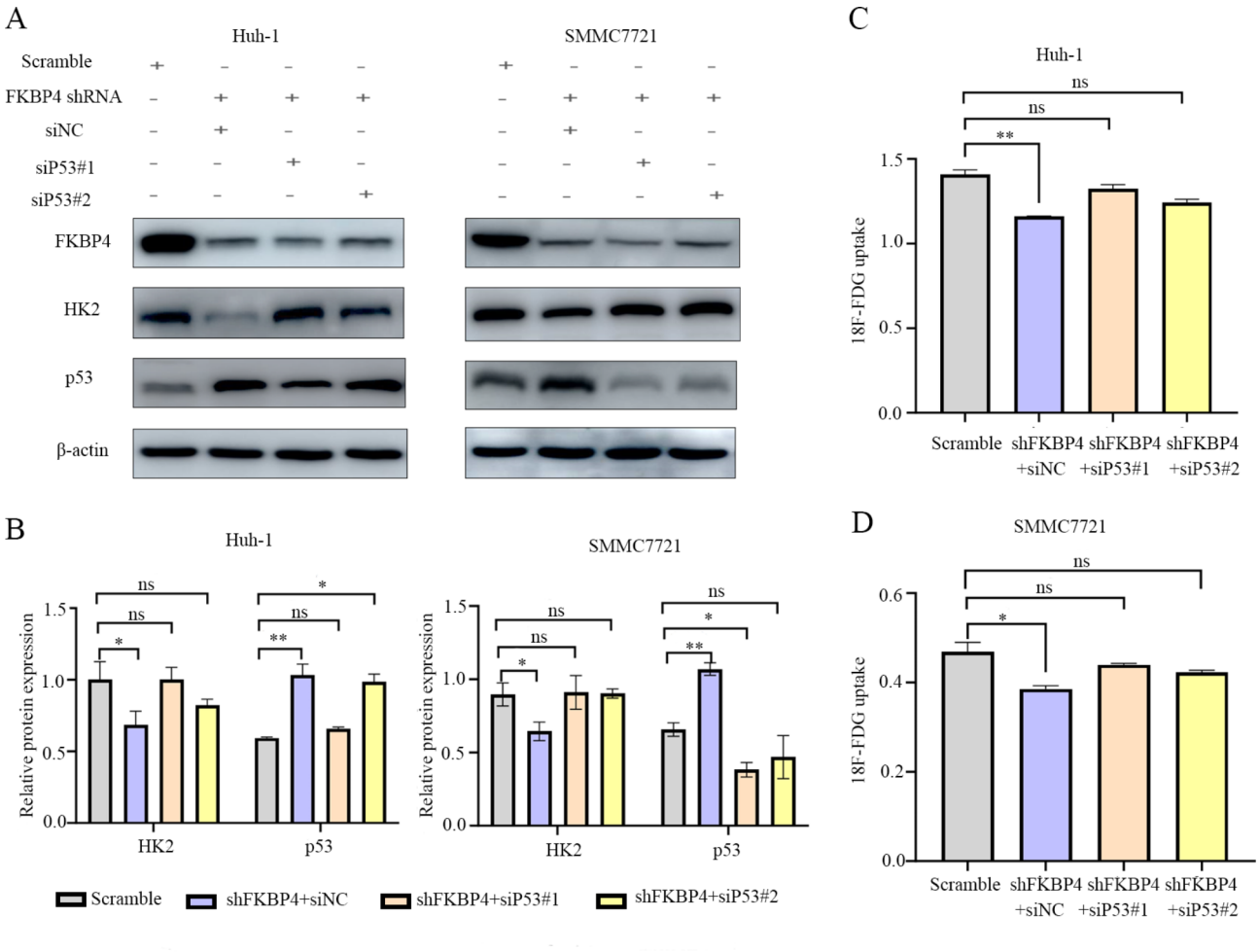


Fig. 7. P53 is a major mediator of FKBP4-induced HK2 activation. The control groups and FKBP4 stable knockdown groups in both Huh-1 and SMMC7721 cells were co-transfected with siRNA negative control (siNC), or p53 siRNA (si-p53#1, and sip53#2). The protein level (**A** and **B**) of anti-FKBP4, anti-HK2, anti-p53, and anti-β-actin were assessed by Western blot analysis. In vitro analysis of ^{18}F-FDG uptake rate in both Huh-1 and SMMC7721 cells after treatment with siNC and sip53#1, and sip53#2 for 48 h. And the ^{18}F-FDG uptake rate (**C** and **D**) was detected using a γ-counter. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

HCC samples, and may act as a putative marker for HCC diagnosis. Additionally, HCC patients expressing higher levels of FKBP4 exhibited worse prognostic outcomes in both TCGA and ICGC cohort. Our research further demonstrated that the expression of FKBP4 is closely related with HCC patients' histologic grade and clinical stage, higher expression of FKBP4 was predominantly found in the higher grade and stage groups. The expression level of FKBP4 was higher than normal hepatocytes in all five HCC cells at the mRNA and protein expression levels in our study. In subsequent CCK-8 assays, cloning assays and transwell assays indicated that blocking FKBP4 was an effective strategy to inhibit HCC cell proliferation. Taken together, our database analysis and related experimental results demonstrated that FKBP4 may function as a potential oncogene in promoting HCC progression. However, further in vivo animal experiments are still required to evaluate the interaction of elevated FKBP4 with HCC risk.

To further explore the impact of FKBP4 changes on HCC, DEGs analysis based on TCGA database was used to enrich the potential signaling pathways associated with abnormal increases of this protein. Subsequently, the gene function enrichment results indicated that the abnormal increase of FKBP4 was enriched in metabolism-related pathways, especially in glycolysis and gluconeogenesis. Changes in metabolic processes represent key drivers of tumorigenesis and malignancy in the liver. Accumulating evidence has highlighted the importance that tumor cells are more likely to utilize aerobic glycolysis for energy requirement instead of oxidative phosphorylation compared with normal cells, even under the condition of physiological oxygen[9,10,17–19]. However, existing literature lacked evidence on FKBPs' regulatory roles concerning these cycles. In our study, based on the TCGA LIHC datasets analyses, we firstly found that several glycolytic enzymes (ENO1, G6PD, HK2, LDHA, PGK1, PKM) were obviously increased in FKBP4 high expression group, this suggests that FKBP4 may play an important role in the glycolysis process. Importantly, we demonstrated that the glucose consumption and lactate production were suppressed in FKBP4 shRNA group compared with scramble control group. Currently,

^{18}F-FDG is the most common clinical PET imaging agent. Based on the glycolysis of tumor cells, ^{18}F-FDG PET is widely used for tumor early detection and diagnosis, evaluation of treatment response, detection of recurrence, etc[20–22]. In the glycolytic pathway, ^{18}F-FDG is transported into cells by GLUT1 where HK2 then transforms it into ^{18}F-FDG-6-phosphate. Cell uptake assay was carried out to further clarify that treatment with FKBP4 shRNA led to a decrease in the uptake of ^{18}F-FDG into HCC cells in vitro. Furthermore, we detect the protein level of these glycolytic related enzymes in HCC cell lines, the downregulation of FKBP4 significantly impaired the expression of HK2. Thus, these data suggested that FKBP4 has a strong impact on aerobic glycolysis and ^{18}F-FDG uptake through interact with HK2. Therefore, FKBP4 inhibitors have the potential to inhibit the tumor glycolysis of hepatocellular carcinoma by inhibiting HK2 levels, as well as affect ^{18}F-FDG uptake.

Mechanistically, many studies have identified HK2 as a pivotal regulator of glucose metabolism, promotes the transformation of glycolysis from oxidative phosphorylation (OXPHOS) to aerobic glycolysis[23,24]. The promoter region of the HK2 gene harbors an abundance of response elements, including STAT3, HIF1, cAMP, and p53, its expression was regulated by these transcription factors[25–29]. As accumulating data support the notion that p53 might confer tumor suppression by inhibiting both glycolysis and OXPHOS, which plays a critical role in cellular glucose metabolism[30]. In alignment with this perspective, our results showed FKBP4 shRNA could enhance the protein level of p53 via extending its half-life in HCC cells. Regulation of P53 protein levels and its functional predominately occurs at the post-translational levels, such as phosphorylation, acetylation, and methylation to enhance its protein stability. Phosphorylation serves as a primary mechanism for stabilizing p53 and enhancing its transcriptional activity. Recent studies reported that phosphorylated of p53 at serine 15 (S15) could lead to p53 stabilization and enhance transactivation of p53 target genes, which might be used as a potential biomarker of gamma-radiation exposure due to the dose-dependent nature of phosphorylation. FKBP4 shRNA-induced p53 upregulation was accompanied by an increase in the phosphorylation levels at S15. Since the S15 residue of p53 is adjacent to the Mdm2 interaction site, its phosphorylation likely diminishes interactions between p53 and MDM2 and contribute to the stabilization of p53[31,32]. Moreover, physiological changes, specifically HK2 protein level and ^{18}F-FDG uptake, induced by FKBP4 shRNA transduction were partially reversed upon transferred p53 siRNA into HCC cells. Our results suggested that FKBP4 could upregulate HK2 by inactivating p53 as well as dephosphorylating phospho-S15 of p53. Taken together, we concluded that FKBP4 shRNA was able to activate p53 by enhancing the protein stability rather than promoting the transcription activity of this crucial tumor suppressor.

In summary, our study finds for the first time that FKBP4 can promote HK2 expression by inactivating p53 as well as dephosphorylating p-S15 of p53, leading to an increased aerobic glycolysis. Besides, our finding highlights the role of FKBP4 serving as an important component involved in the regulation of aerobic glycolysis through a novel FKBP4/p53/HK2 signal pathway. We propose that overexpression of FKBP4 could contribute to tumorigenesis by increase HK2 level through affecting p53 protein stability. Our study reveals a new mechanism by which FKBP4 promotes glycolysis in hepatocellular carcinoma, and finds that FKBP4 is an important regulator of glucose metabolism in HCC cells, providing a new potential therapeutic strategy for targeting glycolysis and tumor growth in this malignancy.

Methods

Data collection

The Cancer Genome Atlas (TCGA) LIHC FPKM data was downloaded from the GDC database (https://portal.gdc.cancer.gov/) and normalized through log2(FPKM + 1). The HCC and adjacent non-tumor tissue samples information were downloaded from the GSE14520, GSE64041 datasets of GEO database (https://www.ncbi.nlm.nih.gov/geo/) and normalized by limma package. Liver Cancer - RIKEN, JP Project from International Cancer Genome Consortium (ICGC LIRI-JP) transcriptomic expression data were downloaded as a validation cohort (https://dcc.icgc.org/projects/LIRI-JP). The different expression genes (DEGs) were analyzed by t-test, and the *p*-value was corrected by FDR. FDR < 0.05 was statistically significant.

Functional enrichment analysis

The "clusterProfiler" R package was utilized to perform GO (Gene Ontology) analysis and the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway according to the methods described in previous studies[33]. The cutoff criterion was defined as $p < 0.05$.

Cell culture and transfection

Human liver cancer cell lines Huh-1, Huh-7, HCCLM3, SMMC7721, HepG2, as well as human normal liver cell LO2, were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, Calif, USA) and 1% penicillin-streptomycin (Solarbio, Beijing, China) at 37 °C in a humidified atmosphere with 5% CO_2.

The small-interfering RNA (siRNA) targeting FKBP4(siFKBP4#1, 5′- GATGATGTCTTCTGGTGTCAT-3′; siFKBP4 #2, 5′- GCATGGAGAAAGGAGAACATT-3′), siRNA for p53(sip53#1, 5′- CGGCGCACAGAG GAAGAGAAT-3′; sip53 #2, 5′- GTCCAGATGAAGCTCCCAGAA −3′) were synthesized by Sangon biotech (Shanghai, China). All transfections were performed with Lipofectamine 3000 (Invitrogen, MA, USA) according to the manufacturer's instructions.

Lentiviruses carrying the FKBP4-RNA interference sequence (sh-FKBP4) were purchased from Gene chem. (Shanghai, China). The stably transfected cells were further selected with puromycin (2 μg/ml) and validated by western blot, then cultured in DMEM medium with puromycin (1 μg/ml).

Quantitative real-time PCR

Total RNA was extracted from indicated cells using TRIzol™ (Takara) reagent according to the protocol, and the RNA was reversely transcribed to cDNA by Super Script First-Strand Synthesis System (Takara). The expression levels of mRNA were quantified using the TB Green® Fast qPCR Mix (Takara).

Western blot

Western blot assays were performed as described previously[34]. In brief, cells were treated for 48 h and then lysed in RIPA lysis buffer containing cocktail protease inhibitors. The protein lysate was separated by SDS-PAGE, and then transferred onto a PVDF membrane. The membrane was then probed with the primary antibody at 4 °C overnight. The corresponding antibodies were shown in Table S1. After washing in TBST for 3 times, the membrane was incubated at room temperature for 1 h with the secondary antibody. Then the levels of protein were detected by chemiluminescence imaging analysis system (Amersham Imager 680, GE, USA).

Cell proliferation, colony-formation assay, and transwell assay

Cells were seeded into 96-well plates at 1.5×10^3 cells/ well, and cultured for 1, 2, 3, 4 and 5 days. and the proliferation of these cells was detected using Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) through measuring the absorbance at the wavelength of 450 nm by the MD SpectraMax i3X Multimode microplate reader (Molecular Devices, CN).

For the colony-formation assay, cells were plated in six-well plates at 1000 cells/well, change the culture medium every 2-3 days. In the second week, colonies were fixed with paraformaldehyde and stained with 0.1% crystal violet.

Transwell assay was used to measure the cell migration ability. Around 100 µL serum-free culture medium containing cells (2×10^5 cells/mL) were seeded into the upper chamber with a pore size of 8 µm (Corning, Costar, USA), in the meantime 800 µL culture medium containing 10% FBS was added in the lower chamber. After 24 h, migrated cells were fixed with paraformaldehyde and stained with crystal violet (Beyotime, CN), then counted with Leica Application Suite V4.12 software under 40× magnification (five views per well). The experiments were performed in triplicate and repeated 3 times.

Glucose consumption and lactate production assay

SMMC7721 and Huh-1 cells (5×10^4 cells/well) were placed into 24-well plates, and maintained for 48 h. Next, medium was collected for detection of glucose consumption and lactate production through Glucose Assay Kit (Nanjing Jiancheng Bioengineering Institute, F006-1-1) or Lactate Assay Kit (Nanjing Jiancheng Bioengineering Institute, A019-1-2) following the in struction's of manufacturer. The level of glucose consumption or lactate production was normalized to control group.

In vitro Fluor-18-deoxy-glucose (^{18}F-FDG) uptake assay

The effect of FKBP4 on the ^{18}F-FDG uptake ability of Huh-1 and SMMC7721 cells was investigated in vitro as described previously[35]. Briefly, scramble cells or FKBP4 shRNA cells were seeded at 3×10^5cells/well in 6-well plates for 48 h, carrying out three replicates for each experiment. For cell uptake studies, 500 µl (37 kBq) of ^{18}F-FDG in DMEM media without FBS was added to three replicate tubes. A tube containing 5 µl (0.37 kBq) of ^{18}F-FDG was prepared as a 1% uptake reference. After incubation at 37 °C for 60 min, three washes were performed using ice-cold phosphate-buffered saline (PBS). Cells were digested with 500 µl 0.05% trypsin, and contents were collected in a plastic tube. The radioactivity of the cells and the reference tube was determined using a γ-counter (CAPINTEC CAPRAC-t, USA).

Tissue microarray (TMA)

A tissue microarray (HLivH060CS01), comprising 29 hepatocellular carcinoma (HCC) tissues and their corresponding adjacent liver samples, along with clinical-pathological parameter data, was procured from Shanghai Outdo Biotech Co., Ltd. The baseline characteristics of the patients represented in the TMA are detailed in Table S2. The tissues underwent incubation with a primary anti-FKBP4 antibody at a dilution of 1:200. Collagen density quantification was conducted using Fiji ImageJ software (https://imagej.net/software/fiji).

Statistical analysis

Statistical analysis was performed using GraphPad prism 7.0 software. Mann-Whitney test and Wilcoxon matched pairs test (two-side) were used to analyze the differences between the two groups. All data were presented as mean ± standard deviation. Differences between variables with p value < 0.05 were statistically significant. * indicated $p < 0.05$, ** indicated $p < 0.01$, *** indicated $p < 0.001$ and *ns* indicated not significant.

Data availability

The datasets analyzed during the current study were derived from the following resources available in the public domain: GDC database (https://portal.gdc.cancer.gov/projects/TCGA-LIHC); GEO database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE14520; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE64041); Liver Cancer - RIKEN, JP Project from International Cancer Genome Consortium (ICGC LIRI-JP) (https://dcc.icgc.org/projects/LIRI-JP). Additional data that support the findings of this study are available on request from the corresponding author, Xingmin Han, upon reasonable request.

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Author contributions

Zhenzhen Zeng: Conceptualization, Methodology, Software, Investigation, Formal Analysis, Writing Original Draft; Shasha Xu: Data Curation, Writing Original Draft; Xingmin Han and Ruihua Wang: Conceptualization, Funding Acquisition, Resources, Supervision, Review & Editing.

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Declarations

Competing interests
The authors declare no competing interests.

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