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High FOXK1 expression correlates with poor outcomes in hepatocellular carcinoma and regulates stemness of hepatocellular carcinoma cells

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ABSTRACT

Aims: Forkhead box (FOX) proteins constitute a huge family of transcriptional regulators, which are involved in a wide range of cancers. FOXK1 is a little studied member of FOXK subfamily. This study aimed to investigate the potential prognostic value of FOXK1 in human hepatocellular carcinoma (HCC) and explore potential underlying mechanisms.

Main methods: We performed bioinformatic analyses to evaluate the prognostic value of FOXK1 expression in human HCC and to reveal the underlying mechanism by which FOXK1 regulates HCC. RT-PCR, FACS analysis and sphere formation assay were carried out to investigate the role of FOXK1 in regulating liver cancer stem cells.

Key findings: Our results demonstrated that FOXK1 was overexpressed in human HCC and positively correlated with cancer progression. DNA hypomethylation and gene copy number variation contributed to the overexpression of FOXK1. Importantly, high FOXK1 expression was associated with both low overall survival probability (OS) and low relapse free survival probability (RFS) of HCC patients. Intriguingly, we found that high FOXK1 expression was correlated with activation of stem cell-regulating pathways in human HCC. Knockdown of FOXK1 resulted in downregulation of the cancer stem cell marker EpCAM and ALDH1 and decreased sphere-forming ability of hepatocellular carcinoma cells.

Significance: Overall, our study identified FOXK1 as a new biomarker for prognosis of HCC patients and revealed its role in regulating stemness of hepatocellular carcinoma cells.

1. Introduction

The first member of the Forkhead box (FOX) family was identified in *Drosophila* about three decades ago [1]. The name of the family was coined because the *forkhead* gene mutation in *Drosophila* led to appearance of ectopic head structures in embryos. The FOX family is a huge transcriptional regulator family consisting of at least fifty family members in human, which are further divided into 19 subfamilies based on their sequence homology [2]. The FOX proteins are defined by an evolutionarily conserved DNA-binding domain (DBD) termed forkhead box or winged helix domain. FOX proteins are involved in a wide range of biological processes, including metabolism, development, differentiation, proliferation, apoptosis, migration, invasion, etc. The roles of FOX proteins in tumorigenesis and cancer progression have been intensively studied [3–10]. Interestingly, despite of the highly conserved forkhead box domain, the roles of FOX proteins in these diseases vary significantly.

FOXK was a little studied subfamily of FOX in the past. In recent years, a number of reports focused on FOXK proteins have been published demonstrating that FOXK1/FOXK2 were involved in tumorigenesis of a wide spectrum of cancers. FOXK2 acts as a tumor suppressor to inhibit the proliferation and invasion of breast cancer cells and to suppress the growth and metastasis of breast cancer in vivo [11]. In breast cancer cells, FOXK2 recruits a set of transcriptional repressive

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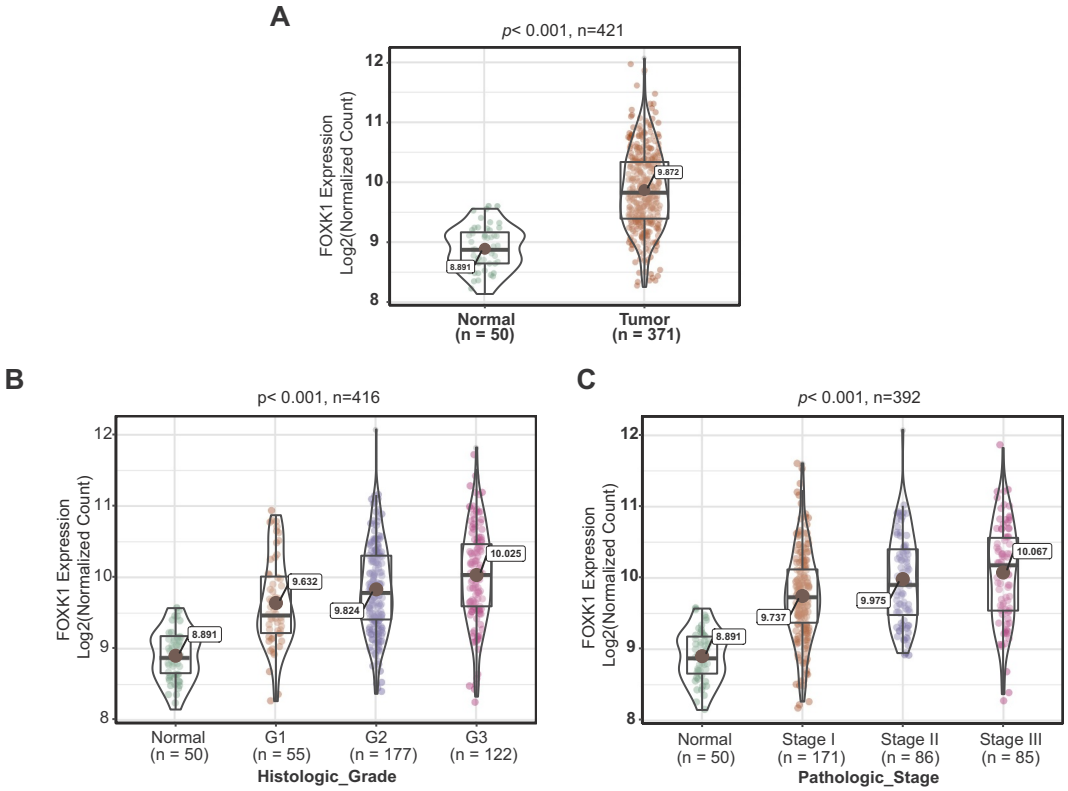

Fig. 1. FOXK1 is overexpressed in human hepatocellular carcinoma. A. Examination of the FOXK1 expression in human hepatocellular carcinoma samples (n = 371) and normal liver tissue samples (n = 50). B, C. FOXK1 expression is positively correlated with cancer progression. Cases lacking histologic grade information or pathologic stage information were eliminated in the corresponding analysis. The case numbers and the mean values of FOXK1 expression (log2) of each group were indicated.

complexes to repress a cohort of genes to regulate the hypoxia response. SUMOylation positively modulates the transcription activity of the FOXK2 [12]. FOXK2 exerts tumor suppressor role in other types of cancer as well, including glioma, clear-cell renal cell carcinoma and non-small cell lung cancer [13–15]. Interestingly, FOXK2 seems to play oncogenic roles as well depending on the cancer types [16,17].

In contrast to the contradictory roles of FOXK2 in tumorigenesis and cancer metastasis, FOXK1 acts unambiguously as an oncogene in human glioma, esophageal cancer, ovarian cancer, colorectal cancer [18–21]. In this study, we assessed the prognostic value of FOXK1 in human hepatocellular carcinomas (HCC) and explored potential underlying mechanisms.

2. Materials and methods

2.1. Gene expression, copy number variation, DNA methylation data source

The data for mRNA expressions (mRNA SeqV2), copy number variation (gene-level), DNA methylation (Methylation450k), and clinical phenotypes of human Liver Hepatocellular Carcinoma were retrieved from The Cancer Genome Atlas (TCGA) project. The detailed clinicopathological data for each sample, including the pathologic stage and histologic grade information, can be accessed via the NIH GDC Data Portal.

The gene expression profile was measured in 371 primary Hepatocellular Carcinoma (HCC) cases and 50 normal liver tissue samples using the Illumina HiSeq 2000 RNA Sequencing platform by the University of North Carolina TCGA genome characterization center. Copy number profile was measured in 370 HCC cases using whole genome microarray and TCGA FIREHOSE pipeline applied GISTIC2 method to produce segmented CNV data, which was then mapped to genes to produce gene-level estimates [22]. DNA methylation profile was measured in 429 samples from HCC cases and normal liver tissue samples using the Illumina Infinium HumanMethylation450 platform. DNA methylation values are recorded for each array probe in each sample via BeadStudio software. Microarray probes are mapped onto the human genome coordinates using xena probeMap derived from GEO GPL13534 record.

2.2. Statistical analyses

The association between FOXK1 expression and the clinicopathological characteristics was assessed using Chi-squared (χ^2) tests. Receiver operating characteristic (ROC) analysis was carried out to ascertain the diagnostic performance of FOXK1 and the optimal cut-off value of FOXK1 expression was determined based on Youden index. The expression levels of FOXK1 were categorized into high and low expressions groups based on the cut-off value. Log-rank test was used to assess the difference between the survival curves. Gene Set Enrichment Analysis (GSEA) was performed using the Bioconductor package clusterProfiler based on KEGG pathways (minimal set size: 20, maximal set size: 500) [22].

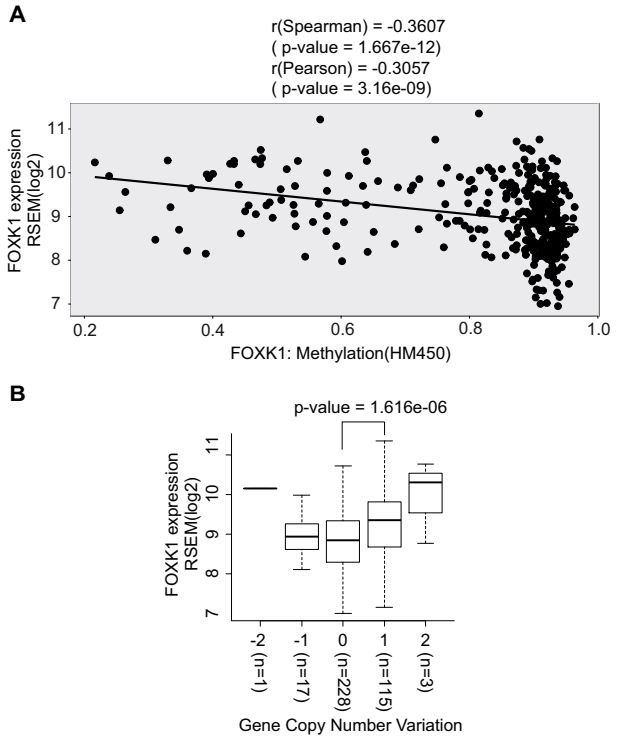

Fig. 2. High FOXK1 expression in human HCC is contributed by DNA hypomethylation and gene copy number variation. A. The correlation between FOXK1 expression and its DNA methylation levels was assessed. The Spearman correlation coefficient and the Pearson correlation coefficient were indicated separately. B. Examination of the effect of gene copy number variation on FOXK1 expression. An additional copy gain for FOXK1 gene was detected in 115 HCC samples.

To evaluate the correlation between DNA methylation and FOXK1 expression, both Spearman correlation coefficient and Pearson correlation coefficient were calculated by R programming using cor.test function. To evaluate the correlation between gene copy number variation and FOXK1 expression, we compared FOXK1 expression between groups (0 and 1). *t*-test was applied to calculate the *p* value.

2.3. Cell culture and gene silencing

Human hepatocellular carcinoma cell line HepG2 was originally obtained from the American Type Culture Collection (ATCC). HepG2 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal calf serum. The cell culture was maintained at 37 °C in a humid atmosphere containing 5% CO_2.

The shRNAs targeting FOXK1 (FOXK1-sh1: GCATGGGCCTGTCCAG CTTCA; FOXK1-sh2: GCCCACATCACCAAGCATTAC) and a negative control shRNA with scrambled sequence (shControl: TTCTCCGAACGT GTCACGT) were constructed into pLV3 lentivirus vector (GenePharma, China). Lentiviruses were produced by the transfection of 293 T cells with plasmids using packaging Mix (GenePharma, China). To knock down FOXK1 expression, HepG2 cells were infected with the lentiviruses and selected with puromycin for 2 weeks.

2.4. RNA extraction, reverse transcription and quantative real-time PCR

Total RNA was extracted from cells with TRIzol (Invitrogen) according to manufacturer's instruction. cDNA was generated by reverse transcription using HiScript III RT SuperMix for qPCR (+g DNA wiper) purchased from Vazyme. Quantative real-time PCR was performed on an ABI-7500 using TB Green™ Premix Ex Taq™ (Tli RNaseH Plus) (TAKARA). Actin was used for normalization of qRT-PCR data.

The following primers were used in the qRT-PCR: FOXK1, sense, 5′-GCTCGGTGGAC TTGAGCAT-3′; FOXK1, antisense, 5′-AAACCGGAA GGTACACTGCT-3′; EpCAM, sense, 5′-AGCGAGTGAGAACCTACT GGA-3′; EpCAM, antisense, 5′-CGCGTTGTGATCTCCT TCTGA-3′; Actin, sense, 5′-CTGTACGCCAACACAGTGCTGTCT-3′; Actin, antisense, 5′-ATCCACATCTGCTGGAAGGTGGAC-3′.

2.5. Sphere formation assay

Cells free from serum were suspended in DMEM/F12 medium supplemented with 1% B27 (Invitrogen), 20 ng/mL epidermal growth factor (EGF), and 20 ng/mL basic fibroblast growth factor (bFGF) were seeded on the ultralow attachment six-well plates (Corning) at a density of 10,000 cells/ml and were grown at 37 °C in a humid atmosphere containing 5% CO_2. Spheres > 50 μm in diameter were counted at day 8.

2.6. Flow cytometry analysis

Single cell suspension was prepared at a concentration of 1.0×10^6 cells in 0.1 mL of PBS containing 2% BSA. The BV421-conjugated anti-EpCAM antibody (Cat. No. 563180, BD Horizon™) was added to the cell suspension, The BV421-conjugated Mouse IgG (Cat. No. 562438, BD Horizon™) was used as negative control. The mixture was subsequently incubated in the dark for 20 min at 4 °C followed by two washes with PBS, The cells were then acquired and analyzed by a flow cytometer.

3. Results

3.1. FOXK1 expression was significantly upregulated in human hepatocellular carcinoma

To assess the role of FOXK1 in hepatocellular carcinoma, we first evaluated the FOXK1 expression levels in human hepatocellular carcinoma (HCC) samples (n = 371) and normal liver tissue samples (n = 50), both obtained from the TCGA project. The results showed that the mRNA level of FOXK1 was significantly higher (approximately 2-fold) in human HCC samples compared to that in normal liver tissue samples (Fig. 1A). We further analyzed the FOXK1 expression in HCC samples of different pathologic stages and histologic grades. Cases lacking histologic grade information or pathologic stage information were eliminated in the corresponding analysis. As shown in Fig. 1B and C, the FOXK1 expression was positively correlated with cancer progression, indicating that FOXK1 may act as an oncogene in human HCC.

3.2. DNA hypomethylation and gene copy number variation contribute to the high FOXK1 expression in human HCC

DNA methylation dysregulation and gene copy number variation are among the common reasons for the gene expression change in cancers. To interrogate the underlying mechanisms for aberrant FOXK1 expression in HCC, we first analyzed the correlation between DNA methylation and gene expression of the FOXK1 gene using the DNA methylation dataset (Methylation450k) combined with the gene

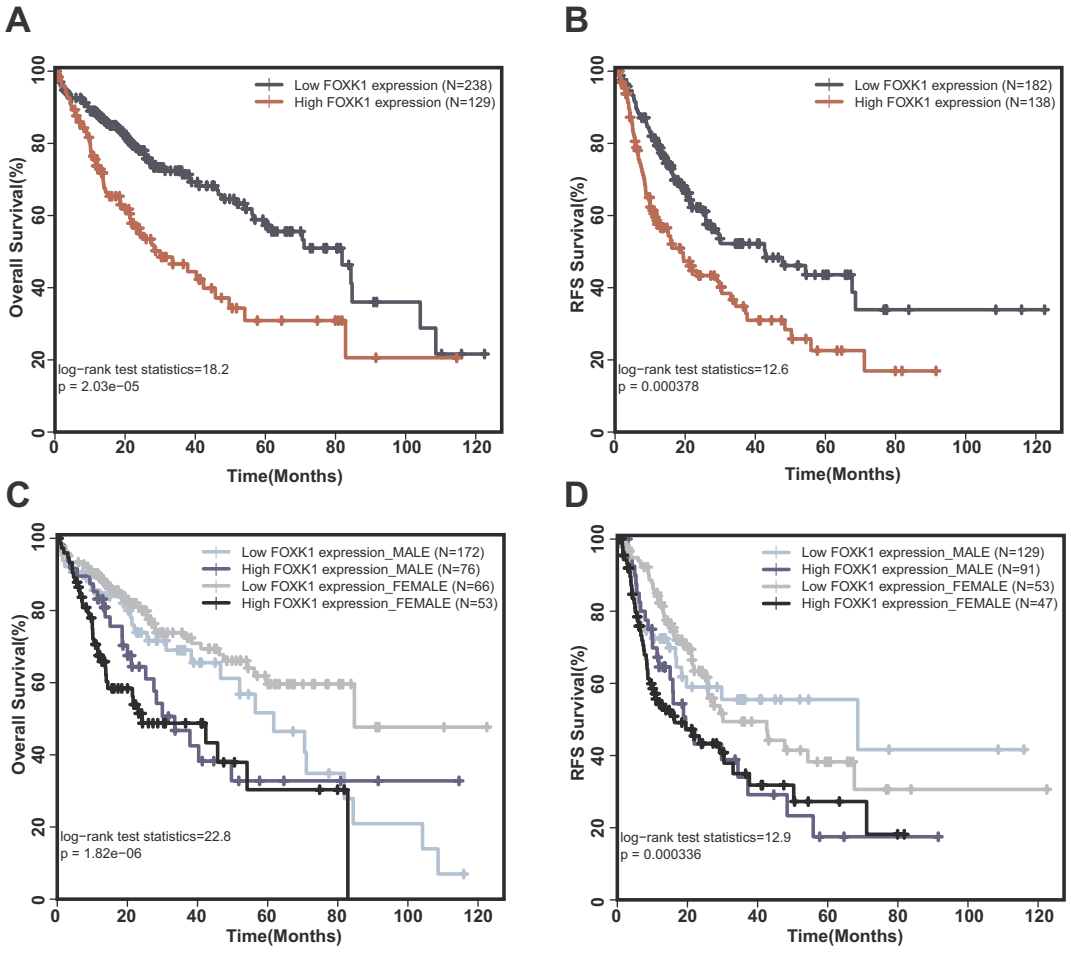

Fig. 3. High FOXK1 expression is associated with poor outcomes of HCC patients. A, B. FOXK1 expression is negatively correlated with both overall survival probability and relapse free survival probability of HCC patients. The expression levels of FOXK1 were categorized into high FOXK1 expression group and low FOXK1 expression group using the optimal cut-off value determined based on Youden index. Log-rank test was performed to assess the difference between the survival curves. The number of cases analyzed in each group is indicated. C, D. FOXK1 expression shows similar correlations with overall survival probability and relapse free survival probability between male and female HCC patients.

expression dataset of human HCC, both obtained from the TCGA project. As shown in Fig. 2A, FOXK1 expression is negatively correlated with its DNA methylation levels, indicating that the DNA hypomethylation is one of the underlying reasons for the overexpression of FOXK1 in HCC.

We next investigated the relationship between the gene copy number variation and the FOXK1 expression. As shown in Fig. 2B, an additional gene copy gain was detected in 115 cases, which led to a significant increase in FOXK1 expression, suggesting that one gene copy gain contributes to the high FOXK1 expression in HCC. However, there was no gene copy number variation detected in most of the HCC cases (n = 228), indicating that an additional gene copy gain is not the main reason for the high FOXK1 overexpression in human HCC.

3.3. High FOXK1 expression correlates with poor overall survival (OS) and relapse free survival (RFS) in patients with HCC

Next, we continued to assess the prognostic value of FOXK1 in HCC. The expression levels of FOXK1 were categorized into high FOXK1 expression group and low FOXK1 expression group using the optimal cut-off value determined based on Youden index. As shown in Fig. 3, high FOXK1 expression was associated with significantly worse overall survival probability (OS) and relapse free survival probability (RFS) in HCC patients.

The associations between FOXK1 expression and the demographic and clinicopathological parameters in HCC patients were investigated via Chi-squared test and were summarized in Table 1. In patients with HCC, FOXK1 expression was correlated with fibrosis ($p < 0.001$) and liver function ($p = 0.004$). Consistent with the results shown in Fig. 1B and C, FOXK1 was positively correlated with histological grade ($p = 0.001$) and pathological stage ($p < 0.001$). We also observed the correlation between gender and FOXK1 expression. The high FOXK1 expression group showed significantly higher proportion of female ($p = 0.009$).

3.4. FOXK1 regulates stemness of hepatocellular carcinoma cells

FOXK1 is a transcriptional regulator which governs the expressions of a cohort of genes. Therefore, high FOXK1 expression may have a profound impact on gene expression program in human HCC. To assess

Table 1
The association between FOXK1 expression and the demographic and clinicopathological parameters of HCC patients.

Parameters	FOXK1 expression		χ^2	*p* Value
	High (N = 129)	Low (N = 238)		
Age (Mean ± SD)	57.82 ± 15.10	60.68 ± 12.18		0.049
Gender				
Female	53	66	6.81	0.009
Male	76	172		
Pathologic stage				
I/II	76	180	13.81	< 0.001
III/IV	45	42		
NA	8	16		
Recurrence status				
0	52	127	2.61	0.106
1	53	88		
NA	24	23		
Living status				
Living	70	167	9.25	< 0.001
Deceased	59	71		
Fibrosis (Ishak score)				
0	24	51	15.68	< 0.001
1–6	33	103		
Null	72	84		
Liver function (Child-Pugh stage)				
A	62	155	10.86	0.004
B/C	8	14		
Null	59	69		
Histologic grade				
G1-G2	67	164	10.36	0.001
G3-G4	60	71		
Null	2	3		
Relative family cancer history				
YES	39	73	0.05	0.821
NO	74	131		
Null	16	34		

this putative impact, we compared the gene expression profiles of the FOXK1_high HCC samples to that of the FOXK1_low HCC samples to identify differentially expressed genes using DESeq2 package, an R/Bioconductor package. As shown in Fig. 4A, we found that most differentially expressed genes (Fold change > 2, *p*-value < 1E-11) were upregulated in FOXK1_high HCC samples, indicating that FOXK1 may act as a transcriptional activator in human HCC. To identify the pathways that were affected by FOXK1 overexpression in human HCC, we performed Gene Set Enrichment Analysis (GSEA) to identify pathways that were altered in the high FOXK1 expression group compared to the low FOXK1 expression group. Interestingly, the result showed that pathways regulating the pluripotency of stem cells were activated in the high FOXK1 expression group (Fig. 4B and C). To further confirm this result, we examined the effect of FOXK1 knockdown on the genes involved in the pathways regulating the pluripotency of stem cells. As shown in Supplementary Fig. S1, the majority of the genes examined were down-regulated by FOXK1 knockdown, suggesting positive involvement of FOXK1 in the pathways regulating the pluripotency of stem cells.

The results of the GSEA analysis prompted us to continue to investigate the role of FOXK1 in regulating stemness of human hepatocellular carcinoma cells. To this end, we knocked down FOXK1 expression in HepG2 cells (Fig. 4D) and carried out sphere formation assay. As shown in Fig. 4E and Fig. 4F, knockdown of FOXK1 resulted in smaller and less spheres formed from HepG2 cells, suggest a positive role of FOXK1 in regulating stemness of hepatocellular carcinoma cells. EpCAM was widely used as an important cancer stem cell marker for hepatocellular carcinoma. We next investigated whether EpCAM expression was regulated by FOXK1. As shown in Fig. 4G, FOXK1 knockdown significantly reduced EpCAM expression. To further confirm our results, we also carried out FACS analysis to examine the effect of FOXK1 knockdown on EpCAM-positive cells. As shown in Fig. 4H, FOXK1 knockdown significantly reduced the percentage of EpCAM-positive cells. Since EpCAM is not the only reported marker for liver cancer stem cells, we went on to investigate the effect of FOXK1 knockdown on other liver cancer stem cell marker genes as well. The result showed that ALDH1 is also significantly down-regulated by FOXK1 knockdown while the expression of CD13 remained unchanged (Fig. 4I). Taken together, our results indicate that FOXK1 regulates stemness of human hepatocellular carcinoma cells probably by regulating EpCAM-positive cells and ALDH1-positive cells.

4. Discussion

Although many *FOX* genes have been reported to be involved in tumorigenesis and cancer progression, *FOXK1* remains a little-studied *FOX* family member in cancer. Here, we assessed the prognostic value of *FOXK1* in human Hepatocellular Carcinoma (HCC) by analyzing combined datasets including gene expression, DNA methylation, gene copy variation and clinical phenotypes obtained from the TCGA project. We found that FOXK1 was overexpressed in HCC samples compared to normal liver tissue samples. We further showed that the expression of FOXK1 was positively correlated with the cancer progression and more importantly, the high FOXK1 expression was associated with poor prognosis of HCC patients. These results are consistent with a previous study which demonstrated that knockdown of FOXK1 inhibited the proliferation, migration and invasion in hepatocellular carcinoma cells [23]. To explore the underlying mechanisms for the oncogenic role of FOXK1 in HCC, we performed GSEA analysis on differentially expressed genes between the high FOXK1 expression groups and the low FOXK1 expression groups. Intriguingly, the results showed that the stem cell-regulating pathways were activated in high FOXK1 expression HCC samples. We further demonstrated that FOXK1 regulated stemness of hepatocellular carcinoma cells.

In most tumors, a subpopulation of tumor cells termed cancer stem cells (CSCs), which exhibit stem cell-like features, are the major reason for the drug resistance in chemotherapy, cancer recurrence and metastasis [24,25]. Our results revealed a positive role of FOXK1 in regulating stemness of human hepatocellular carcinoma possibly via regulating the expression of cancer stem cell marker EpCAM and ALDH1. Our results lead to a better understanding of the regulatory mechanisms of cancer stem cells in hepatocellular carcinoma.

FOXK2, another FOXK subfamily member, has been previously shown to collaborate with multiple transcription co-repressor complexes to suppress the expression of a cohort of genes [11]. By contrast, our results suggest that FOXK1 may act as a transcriptional activator in human hepatocellular carcinoma, indicating that FOXK1 may interact with a different set of transcription regulatory complexes. Further studies are needed to determine the role of FOXK1 in gene transcription regulation and identify its working partners.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.04.068.

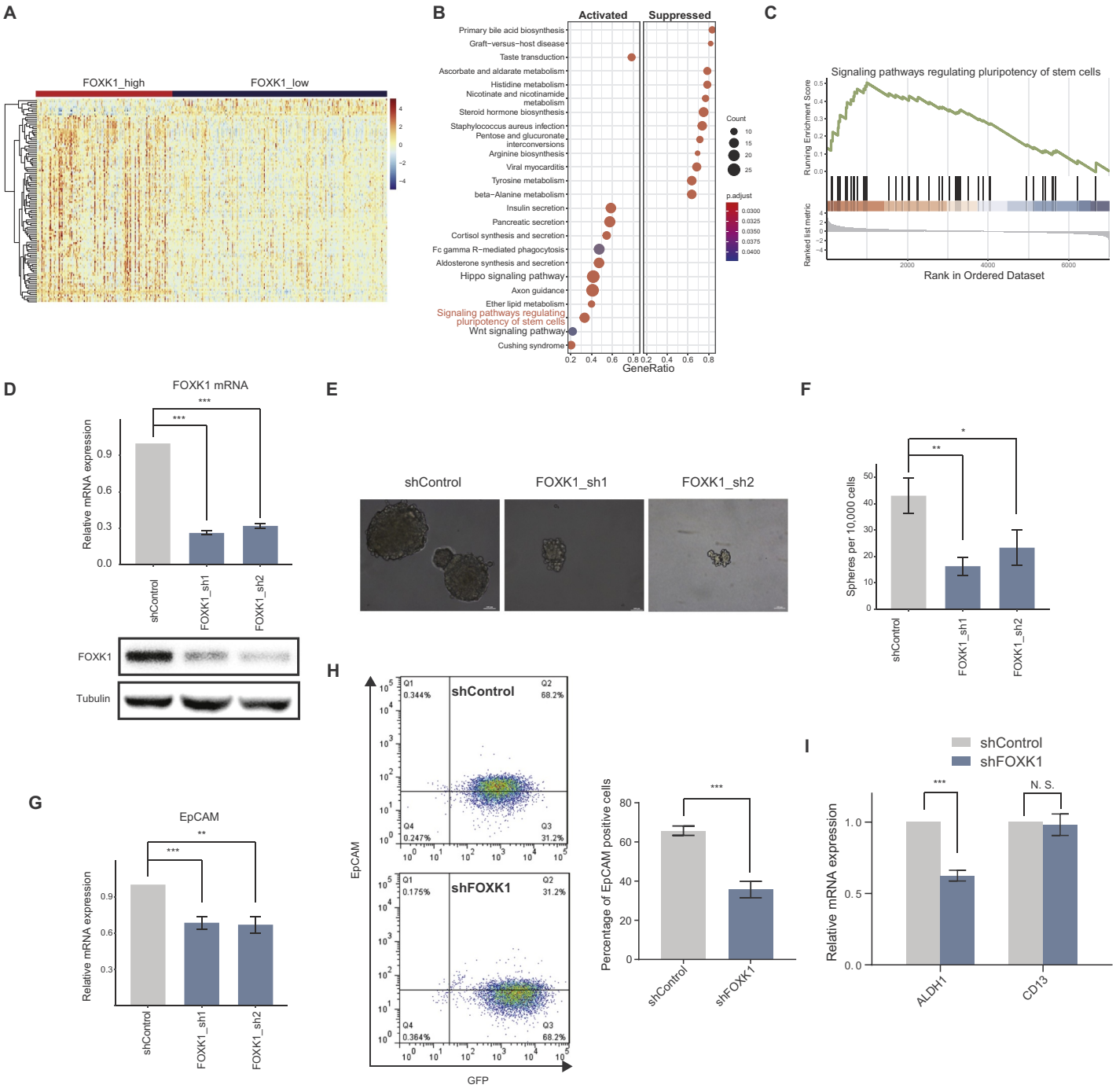

Fig. 4. High FOXK1 expression is correlated with activation of stem cell-regulating pathways in human HCC. A. Hierarchical cluster analysis of differentially expressed genes (Fold change > 2 & $p < 1E-11$) between FOXK1_high and FOXK1_low HCCs. Each cell in the matrix represents the expression of a gene in an individual sample. B, C. GSEA analysis was performed to assess the impact of FOXK1 overexpression in HCC. Of note, stem cell-regulating pathways were activated in FOXK1_high HCC patients. D. qRT-PCR analyses were performed to evaluate the knockdown efficiency of FOXK1. HepG2 cells were infected with lentiviruses to knock down FOXK1 expression. Lentivirus expressing shRNA with a scrambled sequence was used as a negative control. Actin was used as an internal control for normalization. $***p < 0.001$. E. Reprehensive images of spheres formed from HepG2 cells. Scale bar, 100 μm. F. The quantification of numbers of sphere formed from HepG2 cells in three independent experiments. $*p < 0.05$, $**p < 0.01$. G. qRT-PCR analyses were performed to examine the effect of FOXK1 knockdown on cancer stem cell marker EpCAM expression. $**p < 0.01$, $***p < 0.001$. H. FACS analysis was performed to examine the effect of FOXK1 knockdown on EpCAM-positive cells. $***p < 0.001$. I. qRT-PCR analyses were performed to examine the effect of FOXK1 knockdown on the expression of cancer stem cell marker ALDH1 and CD13. $***p < 0.001$.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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Authors' contributions

Haowei Cao and Zhongkun Wang performed experimental work; Daoyong Zhang, Xiaolin Chu, Chuanhui Guo, Simin Shao and Jian Xiao performed bioinformatic analyses; Daoyong Zhang and Junnian Zheng, designed and supervised the study; Daoyong Zhang wrote the manuscript. All authors read and approved the final manuscript.

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