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Original Article

Decreased expression of forkhead box O3 in human hepatocellular carcinoma and its prognostic significance

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Abstract: Background: It has been reported that the transcription factor Forkhead box O3 (FoxO3) was downregulated in various human cancers and associated with poor survival. In the present study, we explored whether FoxO3 could act as a biomarker in hepatocellular carcinoma (HCC). Methods: In the present study, western blot and quantitative real-time PCR (qRT-PCR) were employed in the detection of FoxO3 protein and mRNA expression levels. Immunohistochemical (IHC) was used to detect FoxO3 protein expression in HCC tissues. Kaplan-Meier analysis and Cox proportional hazards regression model were used to analyze the data of FoxO3 expression profile and clinicopathological features. Result: Both protein and mRNA expression levels of FoxO3 were downregulated in HCC. IHC further supported that FoxO3 expression was reduced in HCC tissues. By analyzing the correlation between clinicopathological features and the results of IHC, we found that the low FoxO3 expression were significantly correlated with advanced TNM stage and vein invasion in patients with HCC ($P<0.05$). Kaplan-Meier analysis demonstrated that patients with low FoxO3 expression had a poor overall survival than those patients with high FoxO3 expression. Moreover, multivariate analysis by Cox proportional hazards regression model revealed that the status of FoxO3 expression might be an independent prognostic factor for HCC patients. Conclusion: FoxO3 expression was downregulated in HCC and might serve as a novel prognostic biomarker for HCC patients.

Keywords: Hepatocellular carcinoma, FoxO3, prognosis

Introduction

Hepatocellular carcinoma (HCC) accounts for 80% to 90% of liver cancers and it is the third leading cause of cancer death worldwide [1]. Despite recent advances in clinical and experimental oncology, patients are commonly diagnosed at the advanced stage, and only 30% of patients in Western countries and 10% of patients in Asia are eligible for curative therapies [2]. The long-term prognosis of HCC patients is poor, with a five-year survival rate of <16% [3]. Thus, it is urgent to elucidate the mechanisms underlying HCC at the molecular level and develop novel biomarkers for its early diagnosis and prognosis evaluation.

Tumor growth and metastasis depend on various factors, such as transcription factors. Forkhead box O (FoxO) as transcription factors are the human homologues of the C. Elegans transcription factor DAF-16 [4]. FoxO family members have a highly conserved 110-amino acid DNA binding domain, forkhead box or winged-helix domain and share the characteristic of being regulated by the insulin/Phosphatidylinositol 3-kinase (PI3K)/Protein kinase B (AKT) signaling pathway [5, 6]. The mammalian FoxO transcription factors include FoxO1, FoxO3, and FoxO4 [7]. FoxO members direct transcription of downstream targets involved in cellular responses such as cell cycle arrest/senescence, apoptosis, metabolism, differentiation, and oxidative defense [8]. Recent studies showed that FoxO3 play critical roles in tumor progression. For example, Yang et al showed that PS341 inhibited hepatocellular and colorectal cancer cells through the FoxO3/Catenin Beta1 signaling pathway [9]. Wang et al suggested that FoxO3 could mediate up-regulation of Bim contributed to rhein-induced cancer cell apoptosis

Table 1. Correlation of FoxO3 expression by IHC with clinicalpathological features of HCC

Parameters	Group	Total	FoxO3 expression		*P value*	*Chi square value*
			Low	High		
Gender	Male	62	39 (62.9%)	23 (37.1%)	0.297	1.086
	Female	40	21 (525%)	19 (47.5%)		
Age (years)	<60	56	35 (62.5%)	21 (37.5%)	0.405	0.693
	≥60	46	25 (54.3%)	21 (45.7%)		
Tumor size (cm)	<5 cm	47	27 (57.4%)	20 (42.6%)	0.794	0.068
	≥5 cm	55	33 (60%)	22 (40%)		
Tumour number	Solitary	53	27 (50.9%)	26 (49.1%)	0.093	2.828
	Multiple	49	33 (67.3%)	16 (32.7%)		
HBsAg	Postive	37	19 (51.4%)	18 (48.6%)	0.247	1.338
	Negative	65	41 (63.1%)	24 (36.9%)		
AFP	<20	38	22 (57.9%)	16 (42.1%)	0.883	0.022
	>20	64	38 (59.4%)	26 (40.6%)		
Cirrhosis	Negative	79	50 (63.3%)	29 (36.7%)	0089	2.887
	Positive	23	10 (43.4%)	13 (56.7%)		
TNM stage	I + II	63	30 (47.6%)	33 (52.4%)	0.003	8.54
	III	39	30 (76.9%)	9 (23.1%)		
Vein invasion	Absence	68	34 (50%)	34 (50%)	0.010	6.557
	Presence	34	26 (76.5%)	8 (23.5%)		

AFP: α-fetoprotein.

[10]. Yang et al concluded that Foxo3 activity promoted by non-coding effects of circular RNA and Foxo3 pseudo-gene in the inhibition of tumor growth and angiogenesis [11]. However, the clinical relevance of FoxO3 has not been studied yet, whether FoxO3 expression influence on the prognosis of HCC remains unknown. Thus, in the present study, we explored the expression patterns of FoxO3 in HCC, assessed the relationship between FoxO3 expression and clinicopathological features and prognosis of HCC patients.

Materials and methods

Patients and tissue specimens

Matched fresh HCC specimens and adjacent non-tumor tissues were collected from 102 patients who underwent hepatic resection at The First Affiliated Hospital, and College of Clinical Medicine of Henan University of Science and Technology between June 2007 and July 2010. All specimens were snap frozen in liquid nitrogen immediately following collection and stored at -80°C until use. None of the patients had received chemotherapy or radiotherapy prior to sampling. Clinicopathologic information is summarized in **Table 1**. Overall survival was calculated from the date of initial surgical operation to death or last follow-up. The Research Ethics Committee of The First Affiliated Hospital, and College of Clinical Medicine of Henan University of Science and Technology between approved this study and all patients provided written informed consent.

Cell lines and culture conditions

Three HCC cell lines (Huh7, Hep3B, HepG2), and a normal liver cell line (LO2) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin in humidified air at 37°C with 5% CO_2.

Western blot assay

Tissues and cells protein were immediately collected in a homogenization buffer. Then, these proteins were centrifuged at 13,000 g, 4°C for 20 min. The supernatant was collected. The protein concentrations were analyzed by a BioRad protein assay (Bio-Rad). The protein complexes was diluted in 2 × Sodium dodecyl sulfate (SDS) loading buffer. Then they were boiled. Next, the protein concentration was determined with bicinchoninic acid assay (BCA) protein assay (Pierce Biotechnology). Proteins were divided by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred via polyvinylidene difluoride (PVDF) membranes (Millipore). Membranes were incubated for 1 h with a 1:1000 dilution of the primary antibody and then washed and revealed using secondary antibodies with horseradish peroxidase conjugate (1:5000). Peroxidase was revealed with a GE Healthcare ECL kit (Shanghai, China).

RNA isolation and quantitative real-time PCR

Total RNA was extracted from of HCC tissues or cells using Trizol regent (Invitrogen) and cDNA

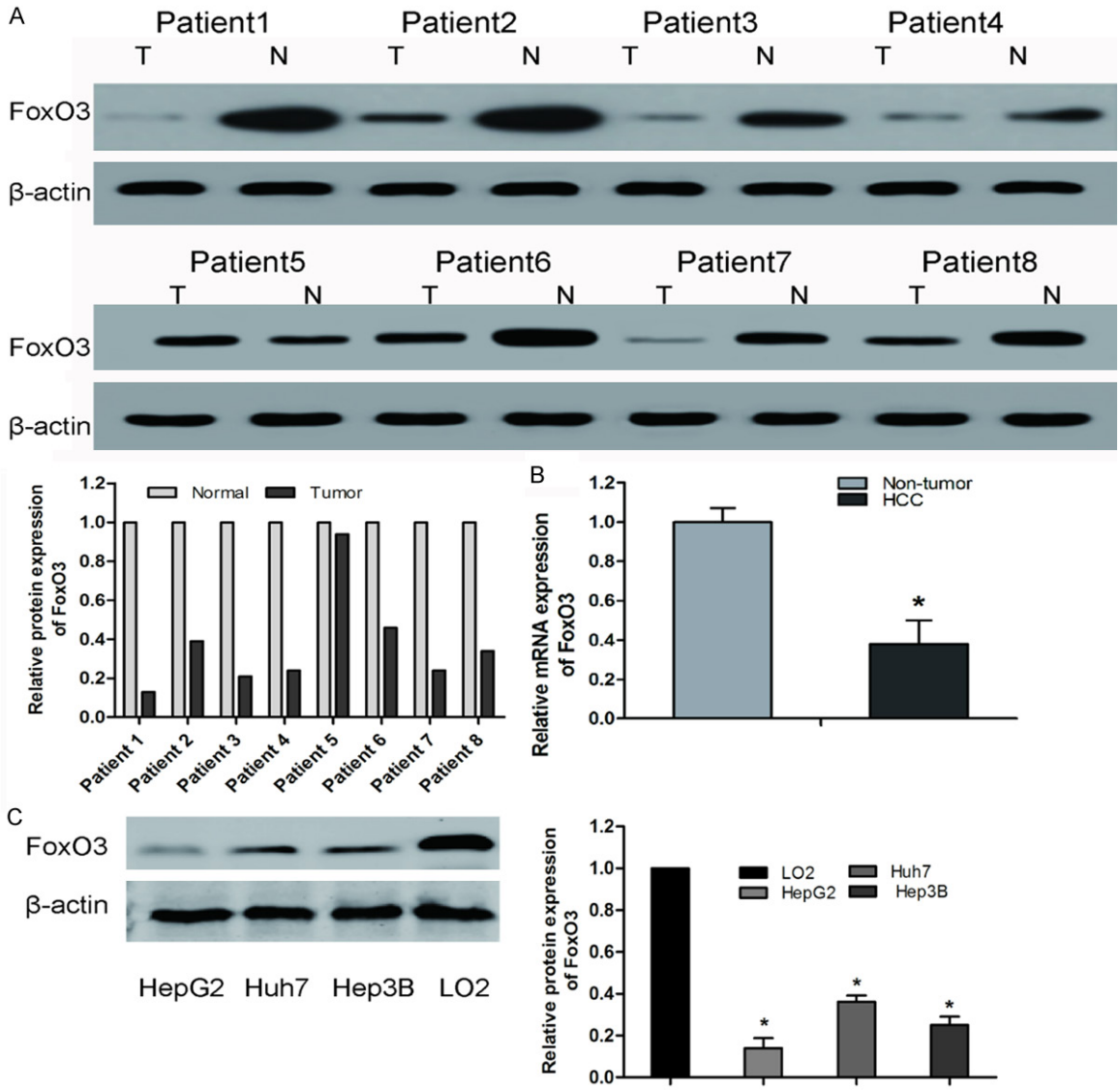

Figure 1. Relative expression of FoxO3 in HCC tissues and cell lines. A. Western blot of 8 paired HCC tissues samples (T) and adjacent non-tumor tissues (N) immunoblotted against FoxO3. B. Expression of FoxO3 mRNA was down-regulated in 30 paired HCC tissues compared with adjacent non-tumor tissues. C. The protein expression of FoxO3 was down-regulated in HepG2, Huh7 and Hep3B cell lines compared with normal liver cell line LO2. *P<0.05.

was synthesized using SuperScript RT kit (Promega) according to the manufacturer's instructions. The expression levels of FoxO3 and β-actin were measured using SYBR green-based real-time PCR performed on the Stratagene M × 3000 P Real-Time PCR system. The primer sequences for FoxO3 were sense, TCACGCACCAATTCTAACGC and anti-sense, CACGGCTTGCTTACTGAAGG, whereas those for β-actin were sense, GATCATTGCTCCTCCTGAGC and anti-sense ACTCCTGCTTGCTGATCCAC. The optimized amplification protocol consisted of an initial denaturation step of 95°C for 10 min, followed by 40 amplification cycles at 95°C for 10 s, annealing at 59°C for 20 s and elongation at 72°C for 10 s. The fold-changes for FoxO3 expression levels were calculated using $2^{-\Delta\Delta Ct}$.

Immunohistochemical analysis

Immunohistochemistry (IHC) was performed using the Histostain-Plus SP kit, which offers superior sensitivity. Briefly, sections were deparaffinized with xylene and rehydrated through gradient ethanol immersion. Endogenous peroxidase activity was quenched by 0.3% (v/v) hydrogen peroxide in methanol for 20 min, fol-

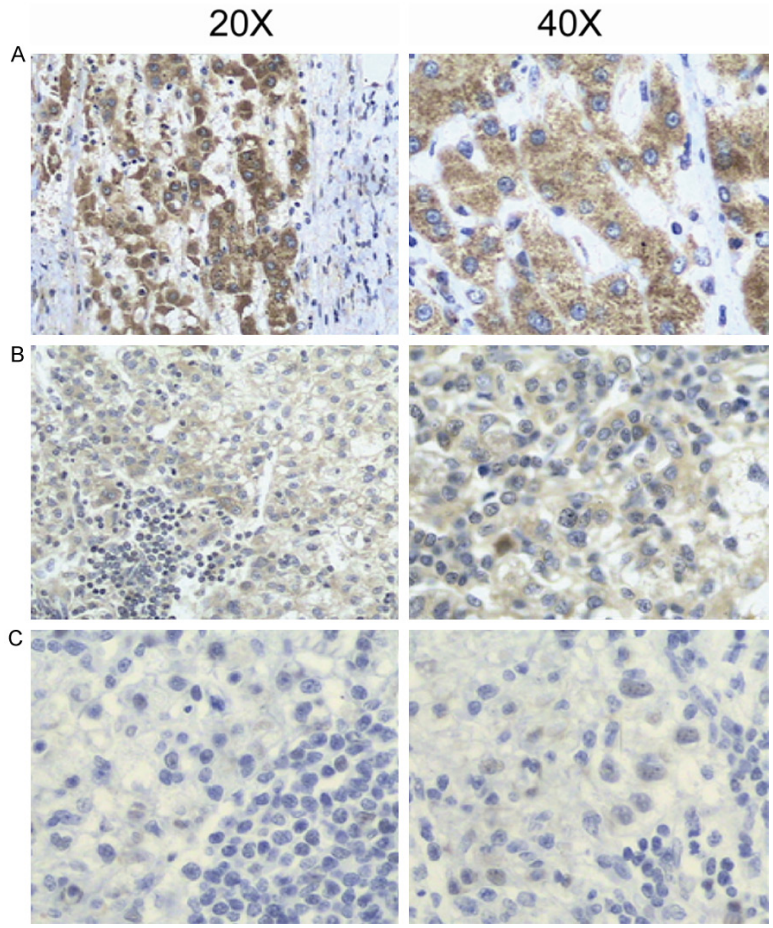

Figure 2. Immunohistochemical analysis of FoxO3 in HCC tissues. Paraffin-embedded tissue sections were stained with FoxO3 antibodies against hematoxylin. The high expression of FoxO3 was detected in well (A) and moderate (B) differentiated histological grade HCC tissues, respectively. Low FoxO3 expression was detected in poorly differentiated (C) histological grade HCC tissues.

lowed by three 5-min washes with Phosphate buffered saline (PBS). The sections were then blocked with 10% (v/v) normal goat serum in PBS for 1 h, followed by overnight incubation at 4°C with the anti-FoxO3 antibody diluted (1:200). A negative control was performed by replacing the primary antibody with pre-immune mouse serum. After three 5 min washes with PBS with Tween 20 (PBST), sections were treated with biotinylated goat anti-mouse antibody for 20 min at room temperature, followed by three additional 5 min washes with PBST. Then, the specimens were incubated with streptavidin-horseradish peroxidase for 20 min at room temperature followed by repeated washes, as indicated above. Reaction product was visualized with DAB at room temperature for 5 min. Sections were counter-stained with haematoxylin for 30 sec and rinsed with tap water, immediately dehydrated by sequential immersion in gradient ethanol and xylene and were then mounted with Permount onto cover slips. Images were generated under a light microscope (Olympus) equipped with a DP70 digital camera.

Evaluation of immunostaining

Sections without the primary antibody were used as negative controls. HCC samples that previously showed immunoreactivity to the FoxO3 antibody were used as positive controls to confirm FoxO3 expression. The slides were evaluated independently by two pathologists who were blinded to the study. Any disagreement was resolved by consensus after joint review. Expression of FoxO3 was evaluated as the percentage of positive cells and staining intensity as previously described. The percentage of positive cells was evaluated quantitatively and scored as 0 for staining of ≤5% of total cells counted, 1. for staining of 5~25%, 2. for staining of 26~50%, 3. for staining of 51~75%, and 4. for staining of >75% of the cells examined. Intensity was graded as follows: 0, no signal; 1. weak; 2. moderate; and 3. strong staining. The two scores were then multiplied to calculate the final score. FoxO3 expression was considered low if the final score was equal to or less than 4; otherwise, FoxO3 expression was considered high.

Statistical analysis

All statistical analysis was carried out using SPSS 18 software and $P<0.05$ was considered statistically significant. A paired Student's t-test was performed to compare the difference of FoxO3 expression between HCC tissues and adjacent non-tumor tissues. The chi-square test was used to determine the correlations of

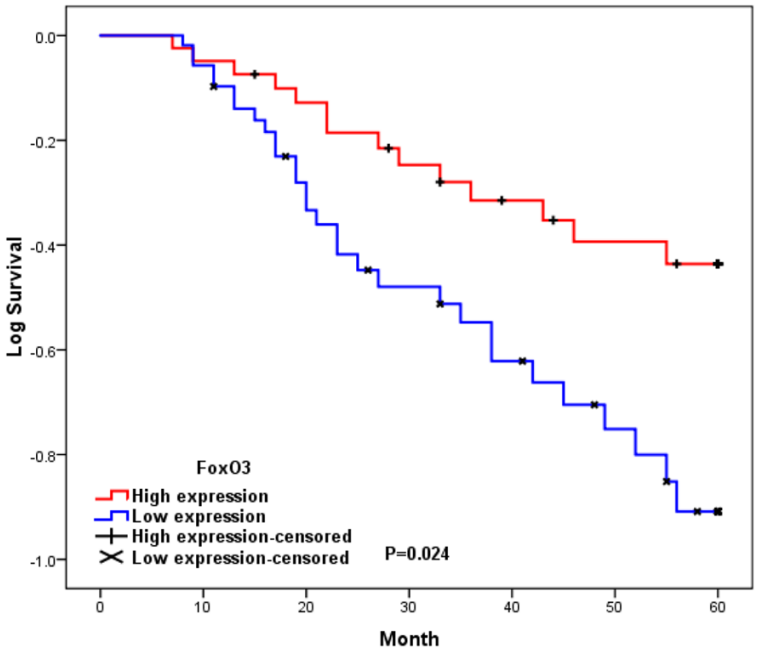

Figure 3. Kaplan-Meier survival curves for HCC patients with high or low expression of FoxO3 (log-rank test, P=0.024).

FoxO3 levels with the clinical factors. Survival curves were drawn by the Kaplan-Meier method and compared by the log-rank test. Cox regression was adopted for multivariate analysis of prognostic factors.

Results

Decreased FoxO3 expression in HCC

In the present study, the FoxO3 expression was first analyzed by Western blot analysis in eight paired of matched HCC tissues and adjacent non-tumor tissues. Representative examples of FoxO3 expression in all tissues were shown in **Figure 1A**, we found that FoxO3 protein was highly expressed in adjacent non-tumor tissues compared to HCC tissues (**Figure 1A**, *P<0.05*). In addition, FoxO3 mRNA expression was measured in 30 paired HCC tissues and adjacent non-tumor tissues by qRT-PCR. We found that FoxO3 mRNA expression levels were significantly decreased in HCC tissues compared with adjacent non-tumor tissues (**Figure 1B**, *P<0.05*). Moreover, we investigated the expression levels of FoxO3 in normal hepatocyte cell line LO2 as well as HCC cell lines (HuH7, HepG2 and Hep3B) by western blot. We found that the expression levels of FoxO3 were increased in LO2 cells compared with HCC cell lines (**Figure 1C**, *P<0.05*). Those findings indicated that FoxO3 might play an important role in the progression of HCC.

Relationship between FoxO3 expression and clinicopathologic features of HCC

In order to explore the prognostic roles of FoxO3 expression, we performed Immunohistochemistry (IHC) analysis in 102 paraffin-embedded HCC sections. The staining intensity of HCC tissues was observed by a light microscope, which demonstrated that the positive protein expression of FoxO3 was manifested as brown or reddish brown granules mainly in the cytoplasm. In addition, IHC study also showed low FoxO3 expression was positively correlated with poor tumor differentiation (**Figure 2**). Among the 102 HCC samples, 60 samples showed low FoxO3 expression, whereas the remaining 42 cases displayed high FoxO3 expression (**Table 1**). The association between FoxO3 expression by IHC and clinicopathological features of HCC was explored by the Chi-square test. Our data showed that low expression of FoxO3 was significantly correlated with advanced TNM stage and vein invasion (**Table 1**, *P<0.05*). However, no significant relationship was found between FoxO3 expression and other clinicopathological features, such as patients' gender, age, tumor size, tumor number, HBsAg, AFP, and liver cirrhosis (**Table 1**, *P>0.05*).

Low FoxO3 expression predicts poor prognosis of HCC patients

In HCC patients, the prognostic significance of FoxO3 was predicted by comparing the high and low expression of FoxO3 for overall survival. Kaplan-Meier survival analysis showed that the overall survival of HCC patients with low FoxO3 expression was poorer than HCC patients with high FoxO3 expression (**Figure 3**, P<0.05). Univariate analysis with the Cox proportional hazards model identified three prognostic factors: TNM stage, vein invasion and

Table 2. Cox regression analysis of features associated with overall survival in 102 HCC patients

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*	HR	95% CI	*P*
Gender	1.418	0.724-2.517	0.559			
Male vs Female						
Age (years)	1.229	0.593-2.178	0.393			
≥60 vs <60						
Tumor size	2.014	0.764-4.191	0.103			
≥5 cm vs <5 cm						
Tumour number	1.683	0.809-3.115	0.198			
Multiple vs Solitary						
HBsAg	1.382	0.791-2.683	0.347			
Postive vs Negative						
AFP	2.471	0.883-5.816	0.094			
>20 vs <20						
Cirrhosis	2.361	0.934-6.211	0.135			
Postive vs Negative						
TNM stage	4.012	1.724-9.975	0.009	3.793	1.603-8.681	0.013
III vs II + I						
Vein invasion	3.724	1.391-11.301	0.004	3.017	1.249-9.936	0.008
Absence vs Presence						
FoxO3	2.907	1.542-8.684	0.001	2.737	1.408-7.769	0.003
Low vs high						

Abbreviations: HR, hazard ratio; CI, confidence interval.

FoxO3 expression (*P<0.05*). The other clinicopathological features, such as patients' gender, age, tumor size, tumor number, HBsAg, AFP and liver cirrhosis were not statistically significant prognosis factors for HCC patients (**Table 2**, *P>0.05)*. Furthermore, multivariate analysis with a Cox proportional hazards model confirmed that low FoxO3 expression was an independent predictor of poor overall survival in patients with HCC (**Table 2**, *P<0.05)*.

Discussion

Hepatocellular carcinoma (HCC) is one of the most malignant solid tumors and the second leading cause of cancer related mortality [12]. The prognosis of HCC is generally poor because of late diagnosis and therapeutic limitations [13]. Therefore, exploration of new molecular biomarkers involved in the progression of HCC could facilitate effective targeted treatment and prognostic assessment. Tumor invasion and metastasis are important issues for understanding tumor biology and further improving the prognosis of patients HCC [14, 15]. This is a very complicated process with multiple promoters or suppressor genes involved. Understanding the genes responsible for either enhancing or suppressing this process would enable novel diagnostic, therapeutic, and prognostic applications to evolve and thus improve the clinical outcome of HCC patients.

Although many human cancers showed decreased expression of FoxO3, the role of FoxO3 in the development and progression of HCC is still unclear. In the present study, we showed that the expression levels of FoxO3 were significantly downregulated in HCC tissues and cell lines compared with adjacent non-tumor tissues and normal liver cell line, indicating that FoxO3 might play an important role in the tumorigenesis of HCC. To further explored whether FoxO3 could accurately predict the outcome in HCC patients, IHC was performed in 102 archived paraffin-embedded HCC samples. Our results reported that low expression levels of FoxO3 in HCC patients were associated with advanced TNM stage and vein invasion. Although some studies have confirmed that downregulation of FoxO3 was associated with poor overall survival of cancers, the prognostic value of FoxO3 expression in HCC remains unclear.

Cho et al showed that lung cancer patients with reduced expression of FoxO3 had a poor over-

all survival than those with high expression of FoxO3 [16]. Similarly, Bullock et al analyzed FoxO3 expression in colorectal cancer samples using IHC and demonstrated that low FoxO3 expression was correlated with more advanced disease stage and shorter disease-free survival in patients [17]. Consistent with these studies, Kaplan-Meier survival analysis showed that the overall survival of HCC patients with low FoxO3 expression was poor than patients with high FoxO3 expression. In addition, multivariate analysis suggested that FoxO3 expression was an independent risk factor in the prognosis of HCC patients. These data demonstrated that FoxO3 might serve as a valuable prognostic biomarker for HCC patients and a potential target for gene therapy in HCC patients.

In summary, our study suggested that FoxO3 play a key role in HCC progression and might provide an opportunity for developing a prognostic biomarker and a novel therapeutic target in the treatment of HCC.

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Disclosure of conflict of interest

None.

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