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Original Paper

Co-Upregulation of 14-3-3ζ and P-Akt is Associated with Oncogenesis and Recurrence of Hepatocellular Carcinoma

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Key Words
14-3-3ζ • P-Akt • Hepatocellular carcinoma • Prognosis

Abstract:
Background/Aims: 14-3-3ζ is involved in the regulation of PI3K/Akt pathway which is closely associated with carcinogenesis. However, the clinical significance of combined detection of 14-3-3ζ and p-Akt in hepatocellular carcinoma (HCC) remains unclear. ***Methods:*** Two-hundred pairs of HCC and adjacent liver specimens were subjected to tissue microarray. The association of 14-3-3ζ and p-Akt levels with the postoperative survival and recurrence in HCC patients was analyzed with univariate and multivariate methods. Moreover, the effects of 14-3-3ζ overexpression on the growth of HCC and the expressions of p-Akt and HIF-1α were assessed in a xenograft mouse model. ***Results:*** Elevated levels of 14-3-3ζ and p-Akt were detected in HCC and a positive correlation between the levels of 14-3-3ζ and p-Akt was verified. HCC patients with satellite nodules, microvascular invasion, portal vein tumor thrombosis, poor tumor differentiation and an advanced tumor stage tended to have higher levels of 14-3-3ζ and p-Akt. In addition, the postoperative 3-, 5-, and 7-year overall survival rates in HCC patients with 14-3-3ζ^{high} and p-Akt^{high} were significantly lower compared with those with 14-3-3ζ^{low} and p-Akt^{low}, and the cumulative recurrence rate in HCC patients with 14-3-3ζ^{high} and p-Akt^{high} was significantly higher than that in those with 14-3-3ζ^{low} and p-Akt^{low}. The multivariate Cox proportional hazard analysis indicated that concomitant upregulation of 14-3-3ζ and p-Akt was an independent factor that predicted poor survival and high recurrence in HCC patients. Furthermore, animal experiment showed that overexpression of 14-3-3ζ accelerated the growth of HCC xenograft tumors and induced the expressions of p-Akt and HIF-1α *in vivo*. ***Conclusion:*** Co-upregulation of 14-3-3ζ and p-Akt predicts poor prognosis in patients with HCC, and 14-3-3ζ-induced activation of the Akt signaling pathway contributes to HCC progression.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent malignant neoplasms and is considered as the third common reason for cancer-related death worldwide [1]. With the development of new diagnostic techniques, early detection of HCC makes it possible to start treatment at an early stage of the disease, which significantly improves the outcome of HCC patients [2]. However, tumor recurrence and distant metastasis still affect numerous HCC patients even after surgical resection or radiofrequency ablation [3]. Therefore, efforts are being made to explore the molecules that are involved in the oncogenesis of HCC for the development of novel diagnostic markers and anti-HCC therapies.

14-3-3 proteins belong to a highly conserved protein family that consists of seven distinct isoforms (β, γ, ε, ζ, η, σ, and τ) in mammals. These proteins exert their functions by directly binding to their target proteins with phospho-serine/threonine motifs. The accumulative evidence shows that the 14-3-3 members play central roles in regulating various biological pathways that control cell cycle, protein trafficking, apoptosis, metabolism, signal transduction, inflammation, and cell adhesion/motility [4-7]. Recent studies reveal that the 14-3-3 proteins are also involved in the pathogenesis of a broad range of diseases, particularly cancers [5, 8-10]. Of types of the 14-3-3 proteins, 14-3-3ζ has been found to promote anchorage-independent growth and proliferation, inhibit apoptosis, and enhance chemo-resistance and metastasis of cancer cells [11, 12]. In addition, a high level of 14-3-3ζ is correlated with poor prognosis of multiple cancers, including breast cancer [13, 14], non-small cell lung cancer [15-17], gastric carcinoma [18], intrahepatic cholangiocarcinoma [19], and HCC [20]. A previous study showed that overexpression of 14-3-3ζ could lead to the activation of Akt in human mammary epithelial cells [21]. Our previous work also demonstrated that 14-3-3ζ overexpression stimulated Akt phosphorylation and further increased HIF-1α expression in HCC cells [22]. It is known that several Akt downstream signaling molecules associated with cell survival are binding partners of 14-3-3 [23-26], and emerging evidence indicates that p-Akt is correlated with poor prognosis of HCC patients [27, 28]. Thus, it is worth evaluating the potential clinical value of co-upregulation of 14-3-3ζ and p-Akt as a prognostic marker in HCC patients.

In the present study, we examined the levels of 14-3-3ζ and p-Akt in HCC specimens using tissue microarrays (TMAs), and analyzed the association between the two molecules and the survival and recurrence in the HCC patients with hepatectomy. Moreover, the effects of 14-3-3ζ overexpression on the growth of HCC and the expressions of p-Akt and HIF-1α were assessed in a HCC xenograft mouse model. Findings outlined in the current study showed that an elevated 14-3-3ζ or p-Akt level was evidently correlated with a lower overall survival (OS) rate and a higher tumor recurrence rate in HCC patients. More importantly, our results indicated that the patients with high levels of both 14-3-3ζ and p-Akt had the worst outcome, suggesting a potential prognostic value of dual detection of 14-3-3ζ and p-Akt for HCC patients.

Materials and Methods

Patients

Patients recruited in this prospective study met the following criteria: the resected lesions were identified as HCC based on pathological results; the patients had not received chemotherapy or radiotherapy before the surgery; absence of distant metastasis; Child-Pugh class A; preoperative performance status of 0-1 according to World Health Organization. Two-hundred qualified HCC patients who underwent curative resection between February 2006 and September 2007 in the Eastern Hepatobiliary Surgery Hospital (Shanghai, China) were enrolled in the study, and the specimens were randomly retrieved to construct TMAs in order to detect the expressions of the indicated proteins. All patients (200/200) had hepatitis B virus background. Tumor stage was determined according to the Barcelona Clinic Liver Cancer (BCLC) staging system. Satellite nodules were defined as small tumors adjacent to the border of the main tumor,

Table 1 Correlation between 14-3-3ζ/p-Akt expression and clinicopathologic characteristics in 200 HCC Patients. Note: The χ^2 test was used for comparisons between groups. HBeAg, hepatitis B e antigen; AFP, alpha-fetoprotein; TBIL, total bilirubin; ALB, albumin; ALT, alanine aminotransferase; ALP, alkaline phosphatase

Clinicopathologic Characteristic	Relative 14-3-3ζ Expression		p value	Relative p-Akt Expression		p value
	Low	High		Low	High	
Age (years)						
≤60	84	78	0.141	87	77	0.066
>60	16	22		13	23	
Gender						
Male	92	90	0.621	89	93	0.323
Female	8	10		11	7	
HBe Ag						
Negative	67	54	0.06	68	55	0.059
Positive	33	46		32	45	
Liver cirrhosis						
No	59	64	0.467	62	61	0.884
Yes	41	36		38	39	
AFP (ng/ml)						
≤20	47	35	0.084	45	37	0.25
>20	53	65		55	63	
TBIL (μM)						
≤17	58	64	0.384	64	58	0.384
>17	42	36		36	42	
ALT (U/L)						
≤45	57	63	0.386	63	60	0.662
>45	43	37		37	40	
ALP (U/L)						
≤92	55	58	0.669	59	54	0.475
>92	45	42		41	46	
Tumor number						
Single	90	81	0.071	86	84	0.692
Multiple	10	19		14	16	
Tumor size (cm)						
≤5	69	60	0.184	73	27	0.012
>5	31	40		56	44	
Encapsulation						
Incomplete	30	27	0.638	27	30	0.638
Complete	70	73		73	70	
Satellite nodules						
No	81	68	0.035	80	66	0.025
Yes	19	32		20	34	
Microvascular invasion						
No	59	41	0.011	57	43	0.048
Yes	41	59		43	57	
PVTT						
No	94	81	0.005	97	78	<0.001
Yes	6	19		3	22	
Tumor differentiation						
I+II	61	46	0.033	67	40	<0.001
III/ IV	39	54		33	60	
BCLC stage						
A	65	51	0.045	70	48	0.002
B+C	35	49		30	52	

and they could only be observed under microscope. Microvascular invasion was defined as tumor spreading to the liver microvasculature but not to the main portal vein. Detailed clinicopathologic features are shown in Table 1. This prospective study was approved by the Ethic Committee of Eastern Hepatobiliary Surgery Hospital and all participants provided their written consents. The data did not contain any information that could identify the patients. All works were undertaken following the provisions of the Declaration of Helsinki.

All patients were followed until September 2014 with the longest follow-up period up to 96 months. The patients were examined every 2-3 months during the first 2 years and every 3-6 months thereafter. The examinations were performed by the physicians who were blinded to the research purpose and study design. The diagnosis of tumor recurrence was based on the cytologic/histologic evidence as well as the noninvasive diagnostic criteria for HCC used by the European Association for the Study of the Liver. Once recurrence was confirmed, treatments were given according to the standard guideline as described [29].

TMA and Immunohistochemistry

Matched pairs of primary HCCs and adjacent liver tissues were used for the construction of TMAs (in collaboration with the Shanghai Biochip Company, Shanghai, China) as previously described [30]. Immunostaining was performed on TMA slides following the routine protocol. Rabbit anti-human 14-3-3ζ polyclonal antibody (ab51129, Abcam) and rabbit anti-human AKT1 (phospho-S473) monoclonal antibody (ab194201, Abcam) were used to detect 14-3-3ζ and p-Akt. The slides were scanned with an Aperio ScanScope GL (Aperio Technologies, Vista, CA), and the protein levels were scored by the Aperio ImageScope software (Aperio Technologies) based on the percentage of positively stained cells and the staining intensity. The scores equal to or higher than the median of all values were defined as high expression (14-3-3ζ^{high} or p-Akt^{high}) while the scores lower than the median were defined as low expression (14-3-3ζ^{low} or p-Akt^{low}).

Cells and animals

Human HCC-LM3 cells were maintained in 10% DMEM supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere consisting of 5% CO_2. BALB/c-nu mice were purchased from Changsheng Biotechnology (Liaoning, China), housed in cages at room temperature (20–25°C) with a constant humidity (55±5%), and provided with food and water *ad libitum*. All the procedures on the animals were approved by the Institutional Animal Ethics Committee and performed in accordance with the Animal Care and Use Guidelines by Eastern Hepatobiliary Surgery Hospital.

Plasmid construction and viral infection

Lentiviruses harboring the 14-3-3ζ expression plasmid or the vector plasmid were constructed by WanleiBio (Shenyang, China), and HCC-LM3 cells were infected with the indicated lentiviral particles at a ratio of 1:50. Cells stably expressing 14-3-3ζ were selected by culturing the cells with puromycin-containing DMEM for one week.

Tumorigenicity assay

Healthy BALB/c-nu nude mice were subcutaneously injected at the right axillary cavity with HCC-LM3 cells (1×10^7/0.1 ml). Then the mice were raised under the same condition for 21 d. The volumes of the solid tumors were recorded every three days since the 7th day after tumor cell inoculation. Upon completion of the assay, all the mice were sacrificed, and the tumors were harvested, weighted and preserved at –80°C.

Western blotting

Total cellular protein was extracted by lysing the cells with RIPA lysis buffer containing 1% PMSF, and the lysates were then centrifugated at 10005×g for 10 min. The concentrations of the protein samples were determined using a Protein Concentration Determination Kit (P0009, Beyotime Technology, Haimen, China) according to the manufacturer's instructions. Forty μg protein was subjected to 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 80 V for 2.5 h, and then transferred onto a polyvinylidene difluoride (PVDF) membrane. After being rinsed with TTBS, the membranes were blocked with skimmed milk solution for 1 h and incubated with the primary antibodies against 14-3-3ζ (1:1000) (#7413, CST, Danvers, MA), p-Akt (1:1000) (#4060, CST), HIF-1α (1:1000) (#3716, CST), and β-actin (1:500) (bsm-33036M, Bioss, Beijing, China) at 4°C overnight. Following four washes with TTBS, secondary horseradish

peroxidase (HRP)-conjugated IgG antibodies (1:5000) (A0208&A0216, Beyotime Technology) were added onto the membranes for 45 min incubation at 37°C. After final six washes with TTBS, the blots were developed using the Beyo ECL Plus reagent (Beyotime Technology) and the images were recorded in the Gel Imaging System. The relative protein levels were calculated by the Gel-Pro-Analyzer (Media Cybernetics, Rockville, MD).

Statistical analysis

Statistical analyses were conducted using the SPSS software (version 19.0; IBM, Armonk, NY). Values are expressed as mean ± standard deviation, and categorical data are represented as frequency distribution. The Student's *t* test was used for comparisons between two groups. One-way ANOVA with *post hoc* multiple comparisons and ANOVA repeated measurements were performed for comparisons among multiple groups. Correlation between 14-3-3ζ and p-Akt was analyzed by the Pearson correlation test. The overall survival (OS) rate and the cumulative recurrence (CR) rate were analyzed as previously described [30]. The differences in OS rates and CR rates were analyzed using log-rank test. The Cox proportional hazard model was used to determine the independent influencing factors on the survival and recurrence based on the variables selected from the univariate analyses. $P < 0.05$ was considered statistically significant.

Results

14-3-3ζ and p-Akt were up-regulated in HCC

The expressions of 14-3-3ζ and p-Akt were examined in 200 primary HCCs and adjacent liver tissues using TMAs. Immunoreactivity to 14-3-3ζ or p-Akt was detected in the cytoplasm of tumor cells (Fig 1A and Fig 1B). The expression levels of 14-3-3ζ and p-Akt were both higher in the HCC tissues as compared with the matched adjacent non-neoplastic tissues (Fig 1A and Fig 1B). Moreover, a positive correlation between 14-3-3ζ and p-Akt expression levels was observed in these clinical samples (r = 0.39, $P = 0.0001$) (Fig 1C).

Association of 14-3-3ζ or p-Akt with clinicopathologic characteristics

The correlation between 14-3-3ζ or p-Akt expression level and the clinicopathologic characteristics of HCC patients was analyzed. As shown in Table 1, a high level of 14-3-3ζ was significantly correlated with the presence of satellite nodules ($P = 0.035$), microvascular invasion ($P = 0.011$), portal vein tumor thrombus (PVTT) ($P = 0.005$), as well as tumor differentiation ($P = 0.033$) and BCLC stage ($P = 0.045$). However, other clinicopathologic characteristics, including age, gender, hepatitis B e antigen (HBe Ag), alpha fetal protein (AFP), total bilirubin (TBIL), alanine aminotransferase (ALT), alkaline phosphatase (ALP), tumor size, tumor number, encapsulation and liver cirrhosis, were not directly related to

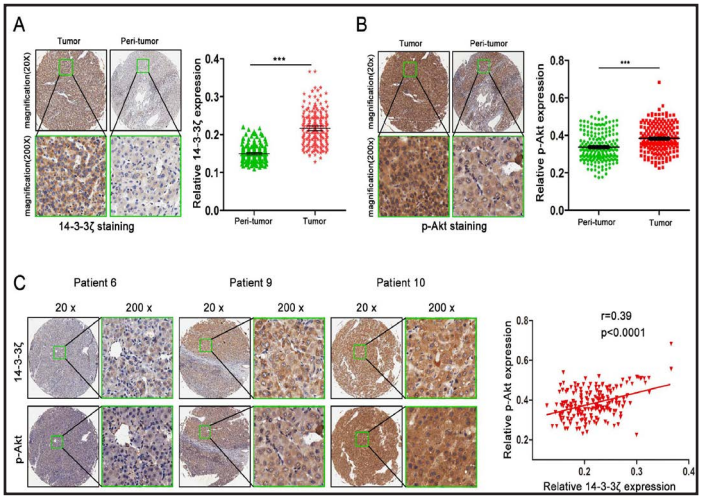

Fig. 1. Increased expressions of 14-3-3ζ and p-Akt in HCC. (A) Relative expression of 14-3-3ζ in HCC specimens and peri-tumor tissues from 200 HCC patients. Representative IHC images are shown in the left panel. *** p<0.001. (B) Relative expression of p-Akt in HCC specimens and peri-tumor tissues from 200 HCC patients. Representative IHC images are shown in the left panel. *** p<0.001. (C) Correlation between 14-3-3ζ and p-Akt expression was examined in 200 HCC specimens (r=0.39, p<0.001). Representative IHC images are shown in the left panel.

the expression level of 14-3-3ζ (Table 1). In addition, an elevated level of p-Akt was significantly correlated with tumor diameter ($P = 0.012$), satellite nodules ($P = 0.025$), microvascular invasion ($P = 0.048$), PVTT ($P < 0.001$), tumor differentiation ($P < 0.001$), and BCLC stage ($P = 0.002$) (Table 1). These results indicated that a high expression level of 14-3-3ζ or p-Akt was associated with the oncogenesis of HCC.

Table 2. Univariate analyses of factors associated with survival and recurrence in HCC patients. † I, 14-3-3ζ^{low}&p-Akt^{low}; II, 14-3-3ζ^{low}&p-Akt^{high}; III, 14-3-3ζ^{high}&p-Akt^{low}; IV, 14-3-3ζ^{high}&p-Akt^{high}

Variable	Overall survival	Time to recurrence
	p value	p value
Age (≤60 vs. >60 years)	0.064	0.098
Gender (male vs. female)	0.354	0.326
HBeAg (negative vs. positive)	<0.001	<0.001
Liver cirrhosis (no vs. yes)	0.498	0.224
AFP (≤20 vs. >20 ng/ml)	0.026	0.130
TBIL (≤17 vs. >17 μM)	0.859	0.189
ALT (≤45 vs. >45 U/L)	0.995	0.724
ALP (≤92 vs. >92 U/L)	0.810	0.940
Tumor number (single vs. multiple)	0.817	0.386
Tumor size (≤5 vs. >5 cm)	<0.001	<0.001
Encapsulation (complete vs. incomplete)	0.643	0.489
Satellite nodules (no vs. yes)	<0.001	<0.001
Microvascular invasion (no vs. yes)	<0.001	<0.001
PVTT (no vs. yes)	<0.001	<0.001
Tumor differentiation (I+II vs. III/ IV)	<0.001	<0.001
BCLC stage (A vs. B+C)	<0.001	<0.001
14-3-3ζ expression (low vs. high)	<0.001	<0.001
p-Akt expression (low vs. high)	<0.001	<0.001
Combined 14-3-3ζ and p-Akt †	<0.001	<0.001
I vs. II	0.459	0.118
I vs. III	0.007	<0.001
I vs. IV	<0.001	<0.001

Association of 14-3-3ζ or combined 14-3-3ζ and p-Akt with the survival and recurrence in HCC patients

The univariate analyses revealed that HBe Ag, serum level of AFP (>400 μg/l), tumor diameter (>5 cm), satellite nodules, microvascular invasion, PVTT, poor tumor differentiation, and an advance BCLC stage were significantly associated with the OS rate and the CR rate in HCC patients (Table 2). The expression of 14-3-3ζ was also correlated with OS and CR rates. The 3-, 5-, and 7-year OS rates in the 14-3-3ζ^{low} group were significantly higher than those in the 14-3-3ζ^{high} group (74% *vs* 47%, 58% *vs* 24%, 46% *vs* 11%, respectively) (Fig 2A). The 3-, 5-, and 7-year CR rates in the 14-3-3ζ^{low} group were significantly lower than those in the 14-3-3ζ^{high} group (48% *vs* 75%, 51% *vs* 82%, 78% *vs* 100%, respectively) (Fig 2B). Moreover, similar results were observed for the influence of p-Akt on the OS and CR rates (Fig 2C and Fig 2D; Table 3). When evaluating the association of combined 14-3-3ζ and p-Akt levels with the outcome of HCC patients, the 3-, 5-, and 7-year OS rates in the 14-3-3ζ^{high}&p-Akt^{high} group were 32.8%, 13.1%, 1.6.%, respectively, which were significantly lower than those in the 14-3-3ζ^{low}&p-Akt^{low} group (Table 2. Fig 2E and Fig 2F; Table 3). The 3-, 5-, 7-year CR rates in the 14-3-3ζ^{high}&p-Akt^{high} group were 88.5%, 90.2% and 100%, respectively, which were significantly higher than those in the 14-3-3ζ^{low}&p-Akt^{low} group (Table 2. Fig 2E and Fig 2F; Table 3).

The individual factors that showed significance in the univariate analyses were further subjected to the multivariate Cox proportional hazard model, and the results indicated that high expressions of 14-3-3ζ and p-Akt were independent factors that significantly affected the OS and CR rates in HCC patients. Moreover, co-upregulation of 14-3-3ζ and p-Akt was

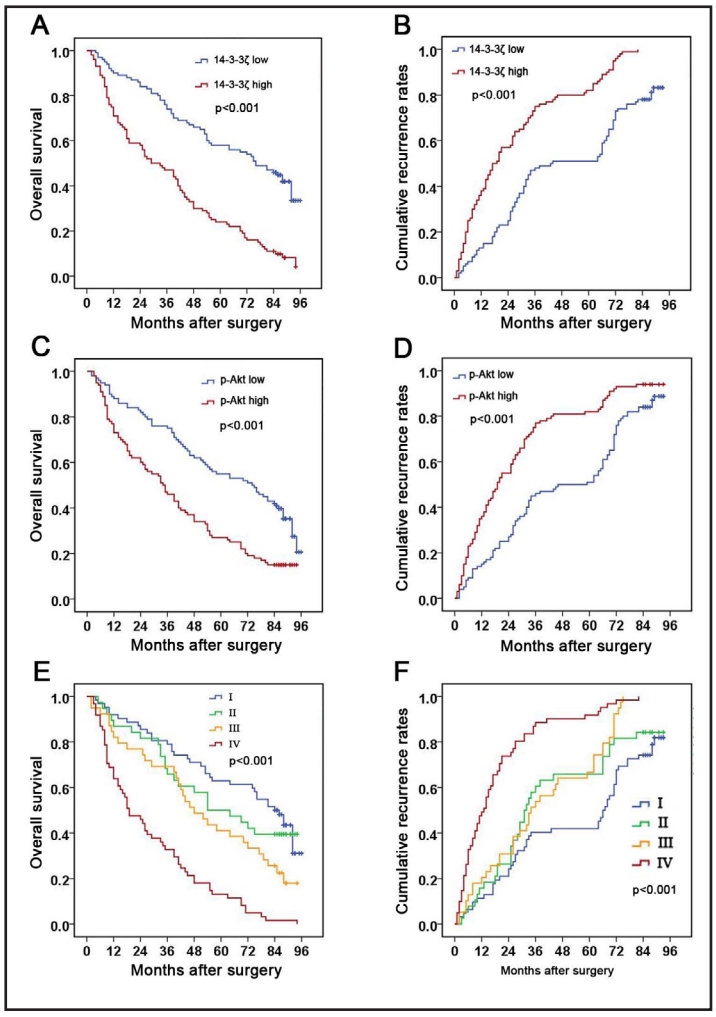

Fig. 2. Co-upregulation of 14-3-3ζ and p-Akt is associated with the worst postoperative outcome in HCC patients. (A&B) The overall survival (A) and cumulative recurrence rates (B) were compared between the 14-3-3ζ^{high} group and the 14-3-3ζ^{low} group. (C&D) The overall survival (C) and cumulative recurrence rates (D) were compared between the p-Akt^{high} group and the p-Akt^{low} group. (E&F) Co-upregulation of 14-3-3ζ and p-Akt was associated with the highest probability of a bad clinical outcome. I, 14-3-3ζ^{low}&p-Akt^{low}; II, 14-3-3ζ^{low}&p-Akt^{high}; III, 14-3-3ζ^{high}&p-Akt^{low}; IV, 14-3-3ζ^{high}&p-Akt^{high}.

also an independent predictor for both survival (P < 0.001) and recurrence (P < 0.001) in HCC patients (Fig 2E and Fig 2F; Table 4).

Effects of 14-3-3ζ overexpression on tumor growth and the expressions of p-Akt and HIF-1α in HCC xenografts

HCC-LM3 cells overexpressing 14-3-3ζ were inoculated in nude mice to generate xenograft tumors. As shown in Fig 3A, compared with the tumors derived parental HCC-LM3 cells or cells infected with lentiviruses only containing the vector, overexpression of 14-3-3ζ accelerated the formation of the xenograft tumors, and pro-growth effect was significant since the 13th day post inoculation (P < 0.05).

Table 3. Cumulative recurrence and survival rates in HCC patients with high/low expression of 14-3-3ζ or/and p-Akt. † I, 14-3-3ζ^{low}&p-Akt^{low}; II, 14-3-3ζ^{low}&p-Akt^{high}; III, 14-3-3ζ^{high}&p-Akt^{low}; IV, 14-3-3ζ^{high}&p-Akt^{high}

	Cumulative recurrence rate			Cumulative survival rate		
	3-year	5-year	7-year	3-year	5-year	7-year
14-3-3ζ expression						
High	75.0%	82.0%	100.0%	47.0%	24.0%	11.0%
Low	48.0%	51.0%	78.0%	74.0%	58.0%	46.0%
p-Akt expression						
High	77.0%	83.0%	93.0%	46.0%	27.0%	16.0%
Low	46.0%	51.0%	82.0%	75.0%	55.0%	45.0%
Combined 14-3-3ζ and p-Akt†						
I	40.3%	41.9%	72.6%	79.0%	62.9%	50.0%
II	60.5%	65.8%	81.6%	65.8%	50.0%	39.5%
III	53.8%	66.7%	100.0%	69.2%	41.0%	25.6%
IV	88.5%	91.2%	100.0%	32.8%	13.1%	1.6%

Moreover, after the 21-day period, the weight of HCC+14-3-3ζ-derived tumors was significantly greater than that of HCC- or HCC+Vector-derived tumors (Fig 3B) ($P < 0.05$). At the molecular level, forced expression of 14-3-3ζ led to increased levels of p-Akt and HIF-1α in the xenograft tumors (Fig 3C), which was in line with our clinical observations and our previous *in vitro* results [31].

Discussion

Great efforts have been made to explore the molecules that are associated with the prognosis of HCC, and accumulating evidence suggests that the use of multiple molecules might be more sensitive than single ones in predicting the progression of HCC [32, 33]. Therefore, in the present study, we investigated the prognostic value of combined detection of 14-3-3ζ and p-Akt in HCC patients. The results showed a positive linear correlation between the expression levels of 14-3-3ζ and p-Akt in HCCs. The HCC patients with higher levels of 14-3-3ζ or p-Akt in the lesion had a shorter survival time and a higher recurrence rate. In addition, the patients with elevated levels of both 14-3-3ζ and p-Akt had even worse outcomes, implying that combined detection of 14-3-3ζ

Table 4. Multivariate analyses of factors associated with survival and recurrence in HCC patients. Abbreviations: HR, hazard ratio; CI, confidence interval; † I, 14-3-3ζ^{low}&p-Akt^{low}; II, 14-3-3ζ^{low}&p-Akt^{high}; III, 14-3-3ζ^{high}&p-Akt^{low}; IV, 14-3-3ζ^{high}&p-Akt^{high}

Variable	OS			Recurrence		
	HR	95% CI	P value	HR	95% CI	P value
HBeAg						
Positive vs. Negative	1.273	0.889-1.823	0.187	1.322	0.942-1.855	0.107
AFP (ng/ml)						
>20 vs. ≤20	1.487	1.035-2.134	0.032			
Tumor diameter (cm)						
>5 vs. ≤5	1.103	0.535-2.276	0.791	1.134	0.572-2.249	0.718
Satellite nodules						
Yes vs. No	0.867	0.585-1.286	0.478	0.932	0.648-1.339	0.703
Microvascular invasion						
Yes vs. No	1.691	1.127-2.537	0.011	1.494	1.044-2.138	0.028
PVTT						
Yes vs. No	3.477	1.944-6.221	<0.001	2.903	1.618-5.208	<0.001
Tumor differentiation						
III/IV vs. I+II	2.092	1.377-3.177	0.001	2.048	1.378-3.044	<0.001
BCLC stage						
B+C vs. A	1.207	0.590-2.471	0.606	1.414	0.715-2.793	0.319
14-3-3ζ expression						
High vs. Low	2.418	1.680-3.479	<0.001	2.359	1.683-3.307	<0.001
p-Akt expression						
High vs. Low	1.401	0.970-2.024	0.072	1.603	1.155-2.224	0.005
Combined 14-3-3ζ and p-Akt†			<0.001			<0.001
I vs. II	0.853	0.494-1.473	0.568	1.175	0.739-1.868	0.495
I vs. III	1.658	1.000-2.748	0.050	1.798	1.136-2.848	0.012
I vs. IV	2.94	1.794-4.817	<0.001	3.407	2.158-5.377	<0.001

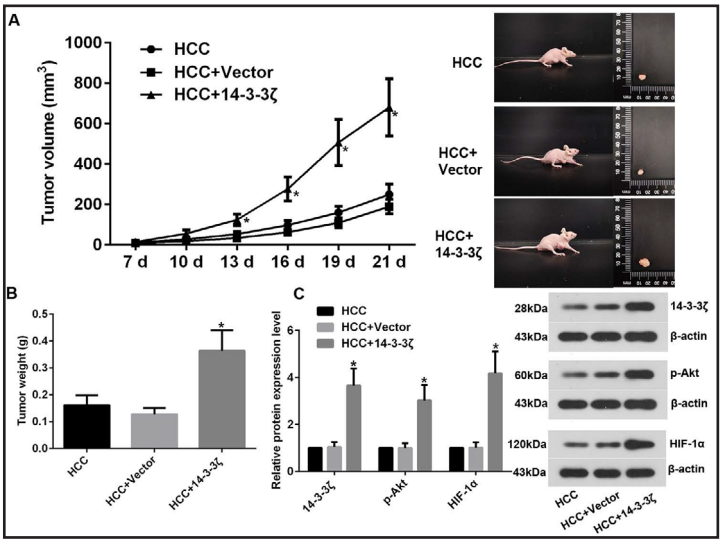

Fig. 3. Effects of 14-3-3ζ overexpression on tumor growth and expressions of p-Akt and HIF-1α in HCC xenograft tumors. Overexpression of 14-3-3ζ increased the growth (A) and weight (B) of HCC xenograft tumors and induced the expressions of p-Akt and HIF-1α in vivo (C). "*", P<0.05 vs. HCC+Vector group.

and p-Akt may be a powerful prognostic factor for HCC. Moreover, the *in vivo* data indicated that overexpression of 14-3-3ζ promoted HCC growth and upregulated the expressions of p-Akt and HIF-1α.

14-3-3ζ is proposed to be a potential prognostic marker and therapeutic target in several cancers [11, 12]. For example, upregulation of 14-3-3ζ in non-small cell lung cancer was significantly associated with recurrence and poor survival of the patients [15]. Neal *et al.* also reported that an elevated level of 14-3-3ζ was associated with reduced survival and increased recurrence in breast cancer [14]. Moreover, Zhang *et al.* reported that 14-3-3ζ overexpression indicated poor prognosis of intrahepatic cholangiocarcinoma in terms of a shorter patient survival and a higher recurrence [19]. In our previous study, we reported that 14-3-3ζ overexpression was significantly correlated with a shorter survival time and a higher recurrence rate in HCC [20]. In the present study, we found that high expression of 14-3-3ζ was associated with an increased recurrence rate and the risk of PVTT formation in HCC patients. These results support 14-3-3ζ as a potential prognostic indicator of recurrence and survival in HCC.

A previous study showed that overexpression of 14-3-3ζ led to hyperactivation of the PI3K/Akt pathway in mammary epithelial cells [21]. The authors further demonstrated that 14-3-3ζ could activate PI3K, the upstream signaling molecule of Akt [34]. Moreover, a number of Akt downstream targets have been identified as the binding partners of 14-3-3ζ [23-26]. For example, 14-3-3ζ directly binds to Akt-phosphorylated Bad, a pro-apoptotic protein, thereby blocking Bcl-2-mediated inhibition of apoptosis [23]. Regarding the pro-tumor function, it is well-recognized that Akt activation is correlated with the clinical outcome of cancer patients [28, 30]. In the present study, a positive linear correlation between the expressions of 14-3-3ζ and p-Akt was observed in HCC. Therefore, we made direct comparisons of the postoperative outcomes among four subgroups, namely 14-3-3ζ^{low}&p-Akt^{low}, 14-3-3ζ^{low}&p-Akt^{high}, 14-3-3ζ^{high}&p-Akt^{low}, and 14-3-3ζ^{high}&p-Akt^{high}, and found that the HCC patients with 14-3-3ζ^{high}&p-Akt^{high} had the poorest outcome in terms of the lowest OS rate and the highest CR rate. Although the expression of 14-3-3ζ was an independent predictor for OS and CR rates in HCC patients, the predictive sensitivity of 14-3-3ζ&p-Akt was higher than that of 14-3-3ζ alone, indicating that combined detection of 14-3-3ζ and p-Akt might be a better method for the prognosis of HCC. Detection of 14-3-3ζ alone or combined evaluation of 14-3-3ζ and p-Akt in HCC provides not only a new approach for the prognosis of HCC but also the potential therapeutic targets for the development of anti-HCC therapy. For example, 14-3-3ζ knockdown was effective in increasing cancer cell sensitivity to cisplatin and postponing tumor growth [17]. In addition, clinical application of specific inhibitors of the Akt pathway is a current focus in the area of targeted cancer therapy [35]. The current study preliminarily demonstrates the association between 14-3-3ζ/p-Akt and HCC progression, yet the values of 14-3-3ζ and p-Akt in HCC prognosis and treatment remain to be further assessed by randomized controlled trials in the future.

Conclusion

In conclusion, the results of the present study highlight that 14-3-3ζ and p-Akt could be potential prognostic markers in HCC and that co-upregulation of the two markers predicts a worse clinical outcome of HCC patients.

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Disclosure Statement

No conflict of interes exists.

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