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ORIGINAL PAPER

The Expression and Prognosis of FOXO3a and Skp2 in Human Hepatocellular Carcinoma

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Abstract The forkhead box proteins (FOXO proteins) comprise a large family of functionally diverse transcription factors involved in cellular proliferation, transformation, differentiation and longevity. Recently, ubiquitination and proteasome degradation of FOXO3a have been reported. In this study, we investigated the role of FOXO3a and Skp2 in human hepatocellular carcinoma progression. Immunohistochemical analysis was performed on formalin-fixed paraffin sections of 91 specimens. Furthermore *in vitro*, western-blot analysis and protein stabilization studies were used to study the relationship between FOXO3a and Skp2. We found that the expression of FOXO3a was negatively related with Skp2 expression ($r=-0.583$; $p<0.05$) and FOXO3a expression correlated significantly with histological grade ($p=0.000$), cirrhosis ($p=0.015$), and tumor size ($p=0.043$) while Skp2 expression correlated significantly with histological grade ($p=0.000$) and tumor size ($p=0.005$). Kaplan-Meier analysis revealed that survival curves of low versus high expressers of FOXO3a and Skp2 showed a highly significant separation in HCC ($p<0.01$). Our results suggested that FOXO3a and Skp2 may be considered to be important prognosis in human hepatocellular carcinoma. In vitro studies suggested that the degradation of FOXO3a may dependent on the expression of Skp2 in the proliferated Huh7 cells.

Keywords Human hepatocellular carcinoma · FOXO protein FOXO3a · S-phase kinase protein (Skp2) · Huh7 · Immunohistochemistry (IHC) · Prognosis

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Introduction

Human hepatocellular carcinoma (HCC) is one of the most common solid tumors in many countries of the world, especially in Asia and Africa, representing the third cause of mortality among deaths from cancer [20]. Many factors such as chronic infections with hepatitis B virus, alcoholic cirrhosis, prolonged dietary exposure to aflatoxin [4], cirrhosis associated with genetic liver diseases [7] were contributed to HCC. However, the principal risk factor varies among countries. Although much is known about both the cellular changes and expressed genes that lead to HCC, the molecular pathogenesis of HCC is not well understood. In addition, a great deal of effort has been devoted to establish a prognostic model for HCC by using clinical information and pathologic classification, which provides information at diagnosis on both survival and

treatment options. Nevertheless, many issues still remain unresolved.

The forkhead box proteins (FOXO proteins) comprise a large family of functionally diverse transcription factors involved in cellular proliferation, transformation, differentiation and longevity [11, 15, 16]. To date, the FOXO family in mammals contains four members: FOXO1 (FKHR), FOXO3a (FKHRL1), FOXO4 (AFX) and FOXO6. These proteins share a high degree of evolutionary conservation, especially in the forkhead DNA-binding domain [1, 3, 8, 14]. Activation of each member of this family in transformed and nontransformed cells results in upregulation of the cyclin-dependent kinase inhibitor $p27^{KIP1}$ and (or) knocked-down of D-type cyclins, thereby arresting cells at G_1 [18, 23]. Activated FOXO proteins also trigger apoptosis in many cancer cell lines through regulation of a number of proapoptotic proteins, including Fas ligand, TRAIL, and Bim [5, 9, 19]. Knocking down the FOXO3a protein in human breast cancer cells or inhibition of the transcriptional activity of FOXO1 in chicken embryo fibroblasts promotes cell transformation and tumor progression [2, 12]. Thus, it has been postulated that FOXO factors play a pivotal role in the inhibition of cell transformation and tumorigenesis. Among these FOXO proteins, FOXO3a is involved in cell transformation, tumor progression and angiogenesis [10, 12, 22].

It has been demonstrated previously that a number of tumor suppressors (e.g., p53, RB, and $p27^{KIP1}$) can be degraded by the ubiquitin pathway in human cancer [24]. Indeed, several tumor suppressor proteins, including $p27^{KIP1}$, p130, and $p57^{KIP2}$, have been shown to be targeted by the F-box motif in Skp2 for degradation Recently, ubiquitination and proteasome degradation of FOXO1 and FOXO3a have been reported [2, 12, 17, 21]. However, the expression of FOXO3a, Skp2 and correlation of FOXO3a and Skp2 in HCC has not been identified. Therefore, in the present study, we examined 91 primary HCCs immunohistochemically and determined the correlation between the levels of these proteins, and various clinical and pathological features including prognosis. In addition, to assess the relationship between FOXO3a and Skp2, Western Blot and protein stabilization studies were performed in the HCC cell line Huh7.

Materials and Methods

Patients and Tissue Samples

HCC tissues were obtained from 91 patients. All underwent hepatic surgical resection without preoperative systemic chemotherapy at the Surgery Department, the Affiliated Hospital of Nantong University. The main clinical and pathologic variables of the patients are shown in Table 1. Seventy-one patients were male and twenty were female; their ages ranged from 32 to 72 years (mean = 51 .37± 10.5). 78 patients were positive for HBV surface antigen, 40 were positive for cirrhosis. Histological grades were classified to well (grade I; n = 31), mood differentiated cancers (grade II; n = 29), and poor differentiated tumors (grade III; n = 31). The follow-up time was 5 years for 74 patients ranging from 1 to 60 months. None of the patients received postoperative adjuvant therapy. Tissue samples were immediately processed after surgical removal. For histological examination, all tumorous and surrounding nontumorous tissue portions were fixed in formalin and embedded in paraffin. Informed consent was obtained from all patients.

Immunohistochemistry (IHC)

Tissue sections (4 μm) were cut, placed on APES-pretreated slides, deparaffinized, rehydrated through graded alcohol and quenched in 3% hydrogen peroxide. Antigen retrieval was performed by microwave heating at high power (750 W) in 10 mM sodium citrate buffer (pH6.0) for three cycles of 5 min each. After blocking with normal serum for 1 h at room temperature, the sections were incubated overnight at 4°C with anti-human FOXO3a rabbit polyclonal antibody (diluted 1:200; Cell Signaling Technology), anti-skp2 mouse monoclonal antibody (diluted 1:100; clone 7B11; Zymed Laboratories , San Francisco, CA., USA). Negative control slides were also processed in parallel using a nonspecific immunoglobulin IgG (Sigma Chemical Co., St. Louis, MO) at the same concentration as the primary antibody. The positive immunostaining of breast carcinoma specimens represented an internal-positive control for preservation of antigenicity in the sections examined. All slides were processed using the peroxidase-antiperoxidase method (Dako, Hamburg, Germany). Diaminobenzidine was used as the final chromogen, and Gill's hematoxylin was used for counterstaining.

All of the immunostained sections were evaluated in a blinded manner without knowledge of the clinical and pathological parameters of the patients. For assessment of FOXO3a and Skp2, five highpower fields in each specimen were selected randomly, and nuclear staining was examined under high power magnification. More than 500 cells were counted to determine the mean percent, which represented the percentage of immunostained cells relative to the total number of cells [25]. In half of the samples, staining was repeated twice to avoid possible technical errors, but similar results were obtained in these samples. The above procedures of evaluation were performed by M.D.L. The obtained results were confirmed by other investigator (Y.W) using a multihead microscope, and a consensus was achieved.

Table 1 FOXO3a、Skp2 expression and clinicopathological parameters in 91 HCC specmens

parametes	total	FOXO3a			Skp2		
		Low ≤0.40	High >0.40	*p*	Low ≤0.24	High >0.24	*p*
Age(yr)							
≤45	29	17	12	0.292	15	14	0.312
>45	62	29	33		39	23	
Gender							
Male	71	34	37	0.339	43	28	0.655
Female	20	12	8		11	9	
Histological grade							
Well	31	6	25	0.000*	29	2	0.000*
Mod	29	14	15		19	10	
Poor	31	26	5		6	25	
metastasis							
Positive	35	17	18	0.765	20	15	0.736
Negative	56	29	27		34	22	
Tumor size(cm)							
≤5	53	37	16	0.042*	38	15	0.005*
>5	38	19	19		16	22	
HBsAg							
(+)	78	40	38	0.732	45	33	0.433
(−)	13	6	7		9	4	
Cirrhosis							
Positive	40	26	14	0.015*	20	20	0.108
Negative	51	20	31		34	17	
AFP水平(ng/ml)							
≤50	44	24	20	0.461	24	20	0.368
>50	47	22	25		30	17	

Statistical analyses were performed by the Pearson χ^2 test. *$P < 0.05$ was considered significant

Cell Culture and Cell Cycle Analysis

Huh7, human hepatocarcinoma cell line, were obtained from the Institute of Cell Biology, Academic Sinica and cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in 5% CO2 at 37°C. Cells were added in 6-well dishes and 24-well dishes 3×10^3 per well. For cell cycle analysis, cells were trypsinized, fixed with methanol, and their nuclei were labeled with propidium iodide as described [6]. 2×10^4 propidium iodide-positive nuclei were gated and analyzed in a FACS/ Calibur Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ USA).

Immunoblot Analysis

Cells were promptly homogenized in a homogenization buffer containing 1 M Tris-HCl pH7.5, 1% Triton X-100, 1% NP-40 (nonidet p-40), 10% sodium dodecyl sulfate (SDS), 0.5% Sodium Deoxycholate, 0.5M EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF, then centrifuged at 10,000 g for 30 min to collect the supernatant. Protein concentrations were determined with a Bio-Rad protein assay (BioRad, Hercules, CA, USA). The supernatant diluted in 2×SDS loading buffer and boiled. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidine diflouride filter (PVDF) membranes (Millipore, Bedford, MA). The membranes were blocked with 5% dried skim milk in TBST (20 mM Tris, 150 mM NaCl, 0.05%Tween-20). After 2 h at room temperature, the filters were washed by TBST for three times and then incubated overnight with polyclonal antibody against using the primary antibodies described below and horseradish peroxidase-linked IgG as the secondary antibodies. Immunoreactive bands were visualized by chemiluminescence (NEN Life Science Products, Boston, MA). Antibodies used were as follows: anti-skp2 (SC-56; 1:1000; Santa Cruz Biotechnology); anti-β-actin (1:4000; Sigma); anti-FOXO3a (1:500; Cell Signal). Finally, horseradish peroxidase-conjugated secondary antibody was added for an additional 2 h and the blots were developed using enhanced chemiluminescence detection system (Pierce). After the chemiluminescence was exposed to X-ray films, the films were scanned using a Molecular Dynamics densitometer (Imaging Technology, Ontario, Canada). Values are responsible for at least three independent reactions.

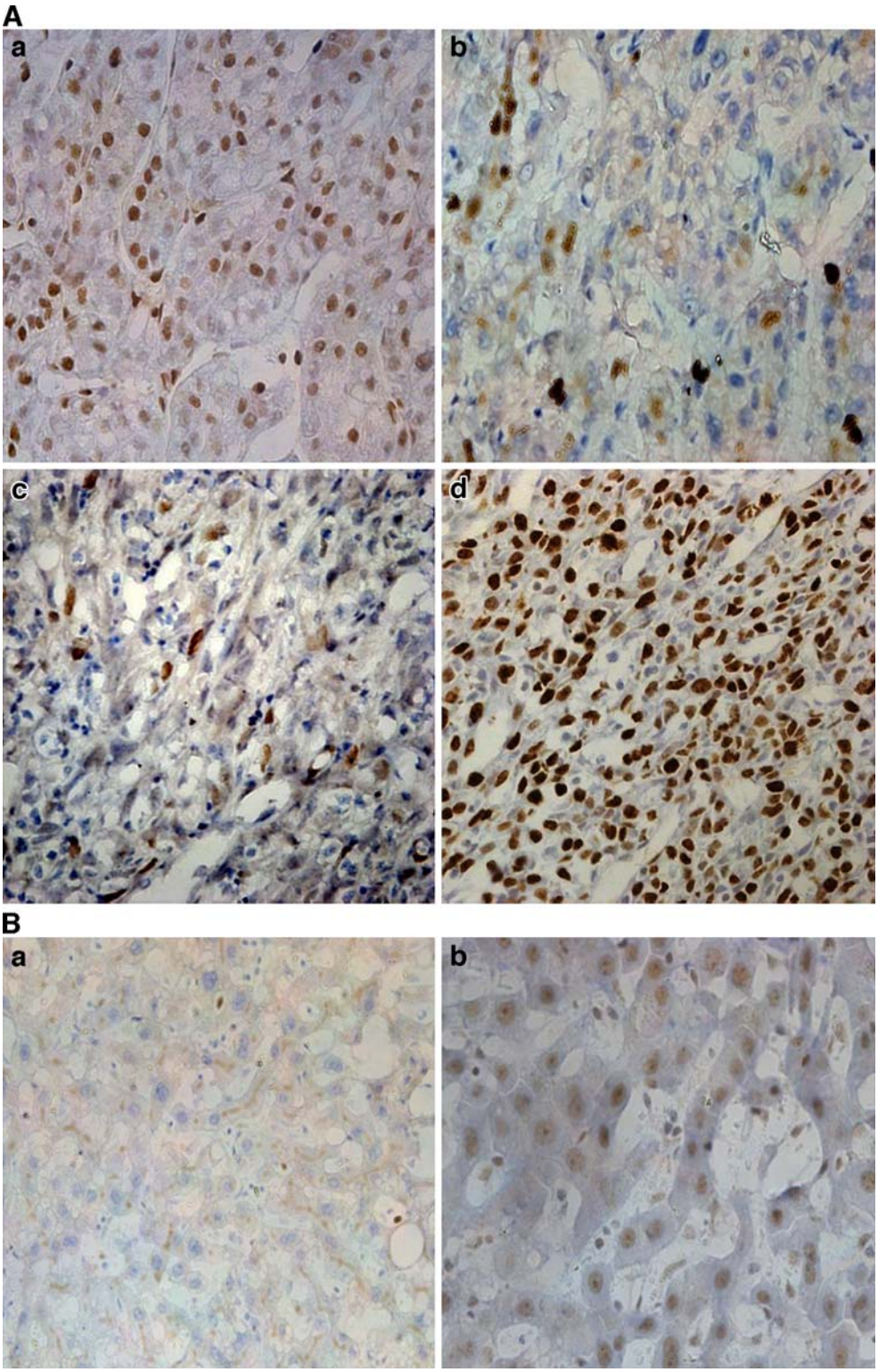

Fig. 1 **a** Low expression of FOXO3a (c) is correlated with high Skp2 (d) in the same HCC specimen while high expression of FOXO3a (a) is correlated with Low Skp2 (b) in the same HCC specimen. (SP×400). **b** The expression of FOXO3a and Skp2 in HCC by immunochemistry. The expression of FOXO3a (a) and Skp2 (b) in the normal liver tissue beside liver carcinoma. (SP×400). **c** Comparison of FOXO3a, Skp2 and PCNA in eight HCC tumors and the adjacent normal tissue tumor cell. Details of the experiments are given in Materials and Methods

FOXO3a Protein Stabilization

For FOXO3a protein stabilization, we measured its half-life by treatment with cycloheximide (cycloheximide, Sigma, St. Louis, MO) for 0, 0.5, 1, 2 and 3 h. Specific proteasome inhibitor MG132 (15 μM) was added to the Huh7 cells. After cycloheximide treatment, cells were collected and the expression of FOXO3a protein was examined by Western blot analysis as described above.

Statistical Analysis

Statistical analysis was performed using the Stat View 5.0 software package. The association between FOXO3a and skp2 expression and clinicopathological features was analyzed using χ^2 test. FOXO3a and skp2 expression in Human hepatocellular carcinoma (HCC) was studied using the Spearman rank correlation test because the data were not normally distributed. For analysis of survival data, Kaplan-Meier curves were constructed, and the log-rank test was performed. Multivariate analysis was performed using Cox's proportional hazards model with $p<0.05$ considered statistically significant. The results of the HCC cells are expressed as the mean ± SE. $p<0.05$ was considered statistically significant.

Results

The Expression of FOXO3a and Skp2 and their Correlation with Clinicopathologic Variables in HCC

We detected the expression of FOXO3a and Skp2 by IHC in 91 HCC samples, and we found that the FOXO3a highly expressed in the adjacent normal tissue while there were no Skp2 expression (Fig. 1b). The typical case showed that low expression of FOXO3a (c) was correlated with high Skp2 (d) in the same HCC specimen while high expression of FOXO3a (a) was correlated with Low Skp2 (b) in the same HCC specimen (Fig. 1a). The results of 91 HCC by IHC are presented in Fig. 1a and summarized in Table 1,

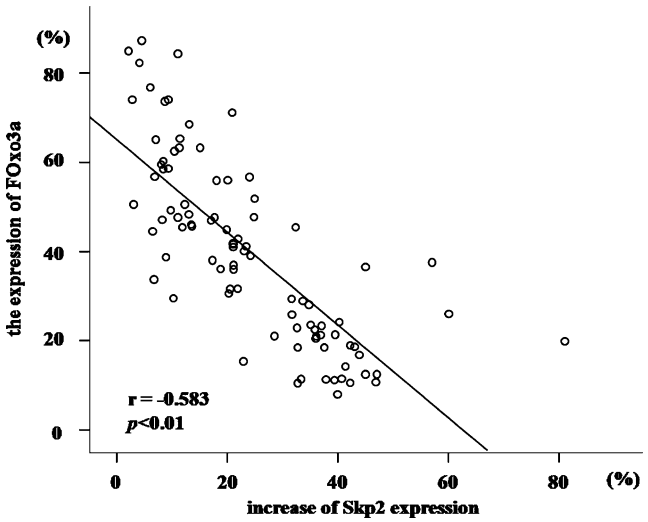

Fig. 2 Relationship between FOXO3a and Skp2 expression in HCC. Scatterplot of FOXO3a versus Skp2 with regression line showing a correlation of the two cell cycle regulators using Spearman's correlation coefficient ($p<0.05$)

the mean percents of FOXO3a and Skp2 were 39.95± 20.50% and 24.16±15.08%, respectively. Based on mean percents, patients were divided into two groups: high FOXO3a expressers (≥ 39.95%) and low FOXO3a expressers (<39.95%) by FOXO3a (Table 1). FOXO3a expression correlates significantly with histological grade ($p=0.000$), cirrhosis ($p=0.015$), and tumor size ($p=0.043$) but there was no relationship between FOXO3a expression and other prognostic factors like tumor metastasis and liver Cirrhosis. Furthermore patients were divided into two groups: high Skp2 expressers (≥24.16%) and low Skp2 expressers (<24.16%) by Skp2. Skp2 expression correlated significantly with histological grade ($p=0.000$) and tumor size

Table 2 Survival status and clinicopathological parameters in 74 HCC specimens

	Total	Survival status		
		Alive	Dead	*P*
Age				
≤45	25	15	10	0.687
>45	49	27	22	
Gender				
Male	61	36	25	0.395
Female	13	6	7	
Histological grade				
Well	26	21	5	0.000
Mod	23	16	7	
Poor	25	5	20	
metastasis				
Negative	28	19	9	0.133
Positive	46	23	23	
Tumor size(cm)				
≤5	43	25	18	0.777
>5	31	17	14	
HBsAg				
Negative	64	38	26	0.250
Positive	10	4	6	
Cirrhosis				
Negative	32	16	16	0.306
Positive	42	26	16	
AFP (ng/ml)				
≤50	38	22	16	0.839
>50	36	20	16	
FOXO3a				
Low expression	37	16	21	0.019
High expression	37	26	11	
Skp2				
Low expression	46	34	12	0.000
High expression	28	8	20	

Statistical analyses were performed by the Pearson χ^2 test. $P<0.05$ was considered significant

(p=0.005), and no significant correlation was found between Skp2 expression and clinicopathologic variables (Table 1).

In most specimens, the proportion of FOXO3a-positive tumor cells was greater than the proportion of Skp2-positive tumor cells. An inverse correlation between FOXO3a expression and Skp2 expression was found, with a correlation co-efficient of−0.583 (p<0.01; Fig. 2).

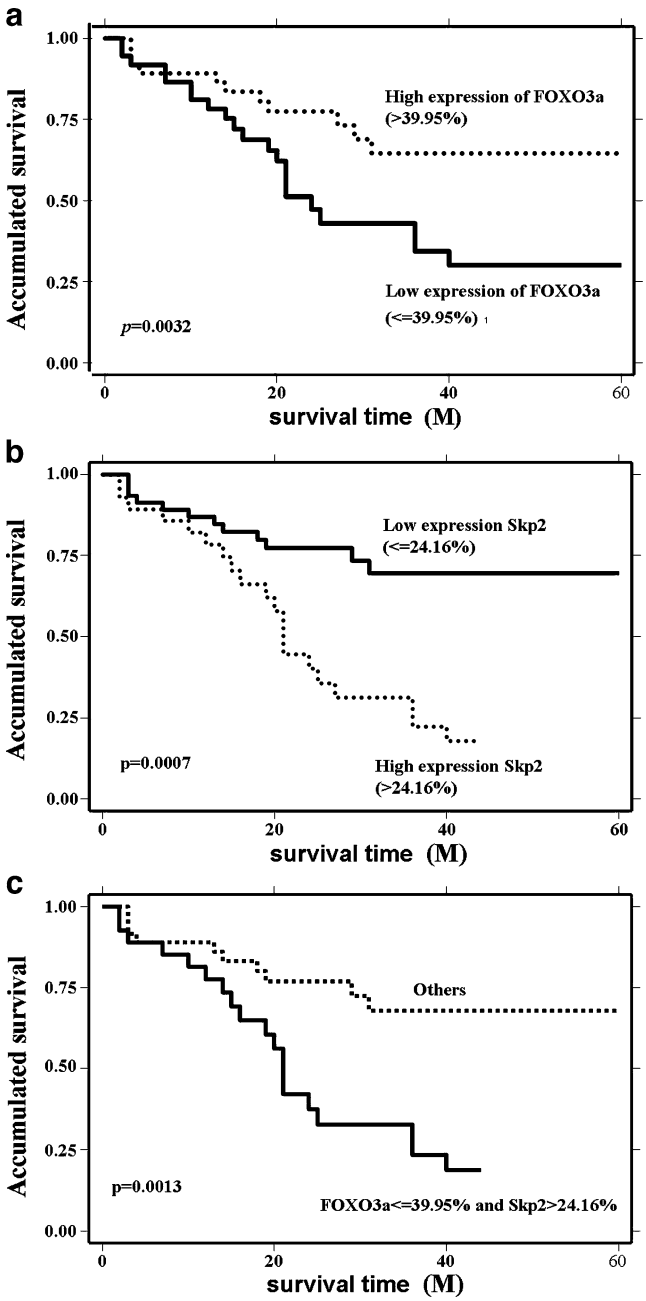

Fig. 3 Accumulated survival curves according to FOXO3a (**a**), Skp2 (**b**) and FOXO3a / Skp2 (**c**) expression

Prognostic Significance of FOXO3a Expression and Skp2 Expression

Concerning survival, only 11 of 37 (29.72%) patients in the high-expresser group died of disease *versus* 21 of 37 (56.75%) in the low-expresser group (Table 2) of FOXO3a expression. When all variables were compared separately to survival status, only Histological grade (p=0.000), FOXO3a (p=0.019), and Skp2 (p=0.000) significantly influenced survival (Table 2). In univariate analysis, the Kaplan-Meier survival curves did not show any significant relationship between tumor metastasis (p=0.0660, log-rank test) or size (p=0.9399, log-rank test) and survival. Conversely, the Kaplan-Meier survival curves of low *versus* high expressers of FOXO3a (Fig. 3a) and Skp2 (Fig. 3b) showed a highly significant separation. Moreover, patients with the phenotype of FOXO3a<=39.95% and Skp2>24.16% had the worst cumulative survival than others (Fig. 3c). When a multivariate Cox proportional hazard model was constructed (including gender, age, tumor grade, tumor size, liver status, and FOXO3a and Skp2 expression), Tumor grade was the strongest independent predictor of survival (p<0.05), the second predictor being FOXO3a and Skp2 (Table 3).

The Expression of Foxo3a Dependent on Cell Cycle

We examined whether the expression of FOXO3a is regulated in a cell cycle-dependent manner. First, the correlation between the cell cycle stages and expression levels of FOXO3a in Huh7 cells was examined. The cell cycle was synchronized at the G_0/G_1 phase by serum deprivation for 72 h, the cells were released and allowed to progress to the S phase by serum stimulus, and the subsequent progress of the cell cycle was monitored by flow cytometry as the time indicated (Fig. 4a). Seventy percent of the cells were arrested at the G_0/G_1 phase by serum deprivation, and the cells progressed from the G_1 to S phase almost synchronously after release. The FOXO3a expression level was high in the G_0/G_1 phase (0 h), declined rapidly at the G_1/S transition point (between 0 and 5 h), and was minimal after about 5 h (Fig. 4b). Of note, the expression of FOXO3a was also accumulated in the MG132-treated cells than the control (Fig. 4c and d).

Discussion

The FOXO family of transcription factors (FOXO1a, FOXO3a, and FOXO4a) regulates diverse cellular responses, including apoptosis, cell cycle arrest, differentiation, DNA repair, and/or oxidative stress (Burgering et al. 2002; Birkenkamp et al. 2003). How FOXO proteins direct such diverse processes is largely unknown, but some of the

Table 3 Contribution of various potential prognostic factors to survival by Cox regression analysis in 74 HCC specimens

	Hazard ratio	95% Confidence interval	*P*
Age(yr)	1.2065	0.5708–2.5503	0.6190
Gender	1.2398	0.5351–2.8727	0.6231
Tumor grade	2.5526	1.5692–4.1523	0.0000
metastasis	2.0090	0.9249–4.3638	0.0660
Tumor size	1.0272	0.5103–2.0678	0.9399
HBsAg	1.9525	0.8001–4.7647	0.1708
Cirrhosis	0.8033	0.4015–1.6072	0.5364
AFP	0.9973	0.4981–1.9967	0.9940
FOXO3a	0.9734	0.9553–0.9918	0.0032
Skp2	3.3532	1.6350–6.8771	0.0007

Statistical analyses were performed by the Pearson χ^2 test. $P<0.05$ was considered significant

cellular alterations associated with skeletal muscle differentiation are similar to those typical of apoptosis. These results manifest that FOXO proteins concerned with apoptosis. We found that FOXO3a expression is correlated with significantly histological grade ($p=0.000$), cirrhosis ($p=0.015$), and tumor size ($p=0.043$). To our knowledge, this is the first reported the FOXO3a expression in HCC.

The ubiquitin-proteasome system is involved in the regulation of a variety of basic cellular processes. Ubiquitination is catalyzed through a multienzyme cascade, including the ubiquitinactivating enzyme (E1), the ubiquitin-conjugating enzyme (E2), and the ubiquitin ligase (E3) (Weissman et al. 2001). The E3 enzymes accomplish the last and most essential step in the conjugation reaction with substrate specificity, and in several cases the target proteins are first marked by phosphorylation for ubiquitination. The F-box protein Skp2 is a positive regulator of G_1-S transition and promotes ubiquitin-mediated proteolysis of the cyclin-dependent kinase inhibitor $p27^{KIP1}$. Its overexpression has been implicated in cell transformation and oncogenesis in both *in vitro* and *in vivo* models. Recent studies investigated that the degradation of FOXO protein is Skp2 dependent ubiquitinatd by proteasome [2, 12, 17, 21]. Skp2 expression is correlated with significantly histological grade ($p=0.000$) and tumor size ($p=0.005$) in our studies. Furthermore the Kaplan-Meier survival curves

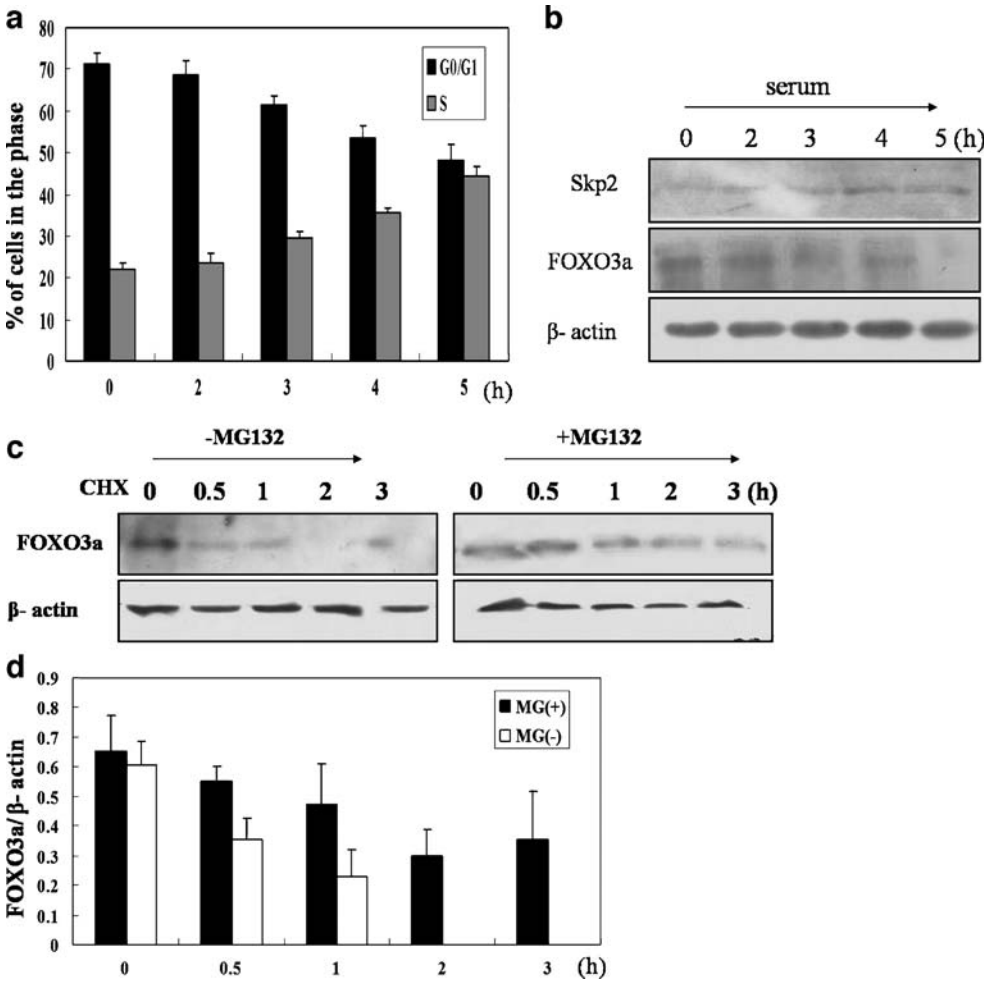

Fig. 4 The expression of Foxo3a dependent on Cell cycle. Cells synchronized at G_1 then progressed into cell cycle by serum after 0, 2, 3, 4, 5 h (**a**). Following the cell cycle progression, most of cells into S phase (a). (**b**) Western immunoblot analysis showing the Foxo3a, Skp2 and β-actin in the Huh7 cells by serum stimulation for 0, 2, 3, 4, 5 h. Foxo3a protein level was high at 0 h, then gradually reduced after serum stimulation. Inversely, the levels of Skp2 was very low at 0 hour, then continuously increased after serum stimulation. Equal loading was confirmed by stripping the blot and reprobing it for β-actin. (**c**) To measure the half-life of FOXO3a protein, we treated cycloheximide (50 ng/mL) for 0, 0.5, 1, 2 and 3 h and then examined the expression of Foxo3a before (left panel) and after (left panel) treatment of MG132 (15 μM) by Western blot analysis. The signal intensity was measured by densitometric scanning and relative expression levels (Foxo3a /β-actin ratio) were presented (**d**)

of low versus high expressers of FOXO3a and Skp2 showed a highly significant separation in HCC ($p<0.01$). When a multivariate Cox proportional hazard model was constructed, Tumor grade was the strongest independent predictor of survival ($p<0.05$), the second predictor being FOXO3a and Skp2 expression.

The expression of the FOXO3a might also be changed on the cell cycle (Fig. 4). Modulation of FOXO3a and Skp2 gene product may also provide a novel target for experimental therapies in HCC. While in vivo, an inverse correlation between FOXO3a expression and Skp2 expression was found, with a correlation coefficient of–0.583 ($p<0.01$; Fig. 2).

Haojie Huang et al [13] demonstrate that Skp2 interacts with and promotes degradation of FOXO protein. Through this mechanism, the tumor suppressor function of FOXO protein, including induction of G_1 arrest and triggering of cell death, is abolished by Skp2 expression. They also demonstrate that loss of FOXO protein is inversely correlated with gain of Skp2 protein in a mouse lymphoma model. They raised that Skp2-mediated degradation and loss of function of FOXO protein can be reversed by proteasome inhibitors, even in the presence of overexpressed Skp2, suggesting that this signaling network is a viable therapeutic target in human cancers, especially those with high levels of Skp2. This is consistent with our results that an inverse correlation between FOXO3a expression and Skp2 expression was found in HCC.

The PI3K/Akt pathway plays a critical role in promoting cell growth and survival downstream of growth factor receptors. Much of the growth and survival activities of Akt have been attributed to the ability of Akt to inhibit the function of proteins that mediate cellular atrophy and programmed cell death. Plas DR, et al [21] using tuberin and FOXO3a as examples, they have shown that Akt targets its substrates for degradation via the proteasome. They propose that proteasomal degradation of substrates may therefore represent a central mechanism by which Akt acts to promote cell growth and survival. Matsuzaki H, et al [17] indicate that insulin-induced export from the nucleus into the cytoplasm as well as phosphorylation is important for efficient FKHR ubiquitination, providing a novel mechanism of ubiquitination of proteins shuttling between the nucleus and the cytoplasm. Given the physiological importance of insulin-dependent ubiquitination, the selective and irreversible degradation of phosphorylated FKHR subsequent to nuclear export may prevent the reentry of FKHR into the nucleus and principally contribute to sustaining the inhibitory effect of insulin on gene expression. Therefore, further studies to identify the E3 enzyme for FKHR would be expected to provide new insights into the mechanism by which insulin regulates FKHR function and gene expression. Thus, how PI3K regulate Foxo3a, phosphorylated Foxo3a and Skp2 dependent degradation in human HCC require our further investigation.

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