RESEARCH ARTICLE

DEP Domain Containing 1 is a Novel Diagnostic Marker and Prognostic Predictor for Hepatocellular Carcinoma

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Abstract

Background: This study was conducted to determine DEPDC1 expression in hepatocelluar carcinomas (HCCs) and to reveal its potential role in diagnosis and prognosis of affected patients. Materials and Methods: DEPDC1 expression at the mRNA level was detected by quantitative real-time PCR (qRT-PCR) in 205 cases of HCC and paired adjacent normal liver tissues, and by semi-quantitative RT-PCR in 20 cases. Survival curves were obtained by using Kaplan-Meier method and Log-rank test. Independent predictors associated with regard to disease free survival (DFS) and overall survival (OS) were identified using the Cox proportional hazard model. Results: High DEPDC1 mRNA levels were detected in 144 out of 205 cases (70.24%) of HCC, significantly associated with clinicopathological parameters, including tumor size (≥4cm), alpha-fetoprotein (≥100ng/ml), B-C of BCLC stage and recurrence. Kaplan-Meier survival analysis revealed that HCC patients with high DEPDC1 expression had poor OS and DFS. Multivariate analysis demonstrated that high DEPDC1 expression was an independent predictor for OS (HR=1.651; 95% 95%CI, 1.041- 2.617; *p*=0.033) and DFS (HR=1.583; 95%CI, 1.01- 2.483; *p*=0.045). Conclusions: Our results indicate DEPDC1 might be a novel diagnostic marker and an independent prognostic predictor for HCC patients.

Keywords: Hepatocellular carcinoma - DEPDC1 - prognosis - diagnosis - biomarker

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Introduction

Hepatocelluar carcinoma (HCC), the sixth most prevalent cancer type, ranks the third leading cause of cancer-related mortality worldwide (Forner et al., 2012), especially in many countries of Asia (Fazeli et al., 2012). Although surgical resection is considered to be the most effective therapeutic method for treatment of patients with primary hepatic carcinoma, the high recurrence rate and early distant metastasis of HCC after surgery remains frustrating (Kobayashi et al., 2006; Tralhao et al., 2007). Identification of factors involved in oncogenesis of HCC may facilitate improvement of early diagnosis and therapeutic approaches (Ji et al., 2014).

DEP domain is a globular domain that consists of approximately 90 amino acids, which was first identified in three proteins: D. melanogaster Dishevelled, C. elegans EGL-10 and mammalian Pleckstrin (Ballon et al., 2006). These proteins are involved in Wnt signaling (Sokol, 2000), G-protein coupled receptor signaling , and signaling in platelets and neutrophils (Kharrat et al., 1998), respectively. DEP domain containing 1 (DEPDC1) is a highly conserved protein, which was reported in bladder cancer cells to act as transcriptional repressor through forming a complex with ZNF224 to suppress A20 transcription, leading to activation of anti-apoptotic pathway through NF-κB activation (Harada et al., 2010). A very recent report showed that, in HeLa and MCF-7 cells, DEPDC1 promotes JNK-dependent degradation of MCL1, an anti-apoptotic Bcl-2 family member, and therefore inhibits apoptosis (Sendoel et al., 2014). Several reports in bladder cancer, breast cancer and lung cancer demonstrated that DEPDC1 up-regulation might have important role in tumorigenesis (Kanehira et al., 2007; Harada et al., 2010; Kretschmer et al., 2011; Okayama et al., 2012; Kassambara et al., 2013). However, whether DEPDC1 also plays a pivotal role in hepatocelluar carcinoma progression and what is its clinical significance in HCC patients are still unknown.

In this study, we detected DEPDC1 expression at mRNA level in HCC tissues, and analyzed the correlation with clinical parameters, as well as the diagnostic and prognostic value.

Materials and Methods

Patients and the source of specimens

A total of 205 cases of HCC samples and paired

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adjacent normal liver tissues were from patients who underwent curative surgical resection from 2001 and 2007 at the Affiliated Hospital of Guilin Medical University. All the cases met the "primary liver cancer clinical diagnosis and staging criteria" and none of them were from patients who received adjuvant chemotherapy or transhepatic arterial embolization. All of them were verified by clinical and pathological examination. The clinicopathological parameters were shown in Table 1. Fresh excised tumor and paired adjacent normal liver tissues for qRT-PCR and reverse-transcription PCR were immediately immersed in liquid nitrogen and stored at -80°C until use. The prognostic data were obtained via follow-up examination. Serum alpha-fetoprotein (AFP) test and ultrosonography (US) scan were conducted every 2 month during the first two years after surgical resection. If the AFP test or US results showed positive outcomes, catscan or magnetic resonance imaging (MRI) was applied to confirm the results. The follow-up process was ended in December 2012, with a median period of 36.0 months (median, 21.0 months, range, 20.0-84.0 months). Disease free survival (DFS) was defined as the period from the date of surgical resection to the date of recurrence, metastasis, death or last follow-up. Overall survival (OS) was defined as the period from the date of surgical resection to the date of death or last follow-up. The study protocol was approved by the Hospital Ethics Committee of Guilin Medical University. Written informed consent based on the 1964 Declaration of Helsinki and amendments was obtained from each patient.

Table 1. Clinical and Biochemical Data of Examined Patients

Parameter	Mean±SD
Age (years)	50.05±11.86
Gender: female/male (n)	30/175
Alcohol abuse: yes/no (n)	107/98
Cirrhosis: yes/no (n)	186/19
HBsAg: negative/positive (n)	37/168
AFP (ng/ml)	3939.21±18817.12
Platelets (10^9/L)	177.17±79.19
Albumin (g/L)	40.08±4.68
TBIL (μmol/L)	18.32±25.26
ALT (U/L)	46.37±44.52
AST (U/L)	56.58±79.65
NLR	2.55±1.86
γGT (U/L)	109.62±97.83

*number of cases (n);hepatitis B surface antigen (HBsAg);alanine aminotransferase (ALT); aspartate aminotransferase (AST); γ-glutamyl transpeptidase (γGT) and total bilirubin (TBIL), Alpha Fetoprotein (AFP); neutrophil to lymphocyte ratio (NLR)

RNA extraction and cDNA synthesis

Total RNA from tumor and paired adjacent normal liver tissues was isolated by Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA concentration was quantified using a spectrophotometer OD260 measurement. Total RNA quality was evaluated by visualization of specific bands (ribosomal RNA, 28s and 18s) on 1.2% agarose gel. Thereafter, total RNA was reverse transcribed to synthesis first strand cDNA using PrimeScript RT reagent Kit (TaKaRa, Otsu, Japan).

Reverse transcription PCR and quantitative real time PCR

The primer premier 5.0 software was used to design the primers for reverse transcription-PCR. Sequences of the DEPDC1 and β-actin primers were verified by PubMed Blast comparative analysis and synthesized by Shanghai Biological Engineering Co., Ltd (Shanghai, China) as follows: DEPDC1: 5'- ACGAAGGTATCCAGAATTG-3' (sense) and 5'-AGATAATACCCAGTGAGGGA-3' (antisense). β-actin: 5'-TCACCCACACTGTGCC CATCT ACGA-3' (sense) and 5'-CAGCGGAACCGCTCAT

Table 2. Correlation between DEPDC1 mRNA Expression and the Clinicopathologic Parameters in HCC

Clinical character	variable	No.of patients	DEPDC1 mRNA low n (%)	DEPDC1 mRNA high n (%)	χ^2	*p* value
Age (years)	<55	140	39 (27.9)	101 (72.1)	0.762	0.383
	≥55	65	22 (33.8)	43 (66.2)		
Gender	Male	175	53 (30.3)	122 (69.7)	0.16	0.689
	Female	30	8 (26.7)	22 (73.3)		
Family history	No	170	49 (28.8)	121 (71.2)	0.414	0.520
	Yes	35	12 (34.3)	23 (65.7)		
HBsAg	Negative	37	11 (29.7)	26 (70.3)	0.000	0.997
	Positive	168	50 (29.8)	118 (70.2)		
alpha-fetoprotein (ng/ml)	<100	85	32 (37.6)	53 (62.4)	4.326	0.038
	≥100	120	29 (24.2)	91 (75.8)		
Median size (cm)	<4	52	22 (42.3)	30 (57.7)	5.251	0.022
	≥4	153	39 (25.5)	114 (74.5)		
Cirrhosis	No	19	4 (21.1)	15 (78.9)	0.759	0.384
	Yes	186	57 (30.6)	129 (69.4)		
Tumor number	Single	140	45 (32.1)	95 (67.9)	1.203	0.273
	Multiple	65	16 (24.6)	49 (75.4)		
BCLC stage	0-A	100	38 (38)	62 (62)	6.348	0.012
	B-C	105	23 (21.9)	82 (78.1)		
Portal vein tumor thrombus	No	164	50 (30.5)	114 (69.5)	0.21	0.647
	Yes	41	11 (26.8)	30 (73.2)		
Recurrence	No	119	44 (37.0)	75 (63.0)	7.072	0.008
	Yes	86	17 (19.8)	69 (80.2)		

*HBsAg; hepatitis B surface antigen; BCLC; barcelona-clinic liver cancer

TGCCAATGG-3' (antisense). The PCR reaction was run for 95°C for 3 min; followed by 35 cycles (for DEPDC1) or 25 cycles (for β-actin) of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s; finally the reaction system was incubated at 70°C for 5 min and terminated at 4°C, products beingvisualized under UV light after ethidium bromide staining on 2% agarose gel.

The sequences of primers for qRT-PCR were as following: DEPDC1: 5'-ACCAAATGTTGGACAAGGCAGC-3' (sense) and 5'-CAGCAAGCTTTGTGTGCCAGTC-3' (antisense); β-actin: 5'-GACAGGATGCAGAAGGAGATTACT-3' (sense) and 5'-TGATCCACATCTGCTGGAAGGT-3' (antisense). qRT-PCR was carried out according to the manuscript of SYBR Premix Ex Taq. cDNA sample mixed with 20 μl Master mix (SYBR® Green PCR Master Mix, Applied Biosystems) was amplified using the ABI Prism 7500 Sequence Detector System Applied Biosystems (Foster City, CA, USA) with the following reactive conditions: incubation at 95°C for 10 min; then 40 cycles of denaturation at 95°C for 2 sec, annealing at 55°C for 5 sec, and extension at 72°C for 15 sec. Relative DEPDC1 mRNA expression was calculated according to our previous report (Liao et al., 2013).

Statistical analysis

Results were analyzed using SPSS version 13.0, p <0.05 was considered statistically significant. The Chi-square (χ^2) test was used to compare the correlation between DEPDC1 expression and clinicopathological parameters, and the Students' t test was used to analysis continuous variables. Kaplan-Meier method was used to plot survival curves, and the log-rank test was used to evaluate the differences in survival curves. Univariate and multivariate regression analysis were performed to identify prognostic factors.

Results

DEPDC1 expression in HCC

To detect the expression of DEPDC1 in HCC patients, we analyzed DEPDC1 mRNA level in 20 cases of HCC tissues and adjacent normal liver tissues by reverse-transcription PCR. The results showed that DEPDC1 mRNA was significantly higher in 14 cases of HCC (Figure 1A). To further confirm RT-PCR results, DEPDC1 mRNA level in 205 cases of HCC was detected by quantitative real-time PCR. The results showed that DEPDC1 mRNA was up-regulated in 144 cases (70.24%), but down-regulated in 51 cases (29.76%) compared with adjacent normal liver tissues. The expression of DEPDC1 in HCC tissues was significantly higher than that in adjacent normal liver tissues (-9.96±0.2254 *vs*-14.50±0.2104, p<0.0001) (Figure 1B).

To assess the diagnostic value of DEPDC1, we compared serum AFP level and DEPDC1 in each patient. Interestingly, we found that the increase of serum AFP level was not always accompanied by DEPDC1 mRNA up-regulation. As shown in Figure 1C, AFP alone was increased in 27 cases. DEPDC1 mRNA alone was

Table 3. Association between DEPDC1 Expression, Clinical Parameters and Disease-free Survival/Overall Survival.

Clinical character	Category	No.of patients	Disease-free survival (months)			Overall survival (months)		
			Mean	95% CI	*p* value	Mean	95% CI	*p* value
DEPDC1 expression	Low	61	55.32	46.88-63.76	0.002	60.24	52.74-67.74	0.004
	High	144	38.82	33.36-44.28	45.86	40.77-50.95		
Age (years)	<55	140	42.90	37.18-48.62	0.453	48.71	43.41-54.01	0.393
	≥55	65	46.17	38.04-54.30	53.43	46.09-60.76		
Gender	Female	175	49.02	38.47-59.57	0.095	58.14	47.05-69.23	0.154
	Male	30	42.41	37.42-47.41	48.82	44.18-53.47		
Family history	No	170	41.84	36.77-46.92	0.053	48.35	43.66-53.04	0.055
	Yes	35	55.78	44.35-67.22	59.21	48.88-69.54		
HBsAg	Negative	37	40.98	29.65-52.30	0.621	49.32	39.07-59.56	0.832
	Positive	168	44.64	39.47-49.80	50.44	45.69-55.20		
AFP (ng/mL)	<100	85	49.49	42.23-56.75	0.047	56.02	49.66-62.38	0.040
	≥100	120	40.28	34.19-46.37	46.13	40.42-51.84		
Tumor size (cm)	<4	52	65.77	58.09-73.45	p<0.001	70.18	63.88-76.47	p<0.001
	≥4	153	36.41	31.19-41.64	43.52	38.59-48.45		
Cirrhosis	No	19	39.87	24.88-54.86	0.605	44.32	30.36-58.27	0.462
	Yes	186	44.48	39.53-49.43	50.79	46.27-55.312		
Tumor number	Single	140	49.24	43.44-55.05	0.002	54.93	49.74-60.12	0.001
	Multiple	65	32.48	25.70-39.27	40.05	32.90-47.20		
BCLC stage	0-A	100	57.08	50.37-63.78	p<0.001	62.62	56.99-68.26	p<0.001
	B-C	105	32.22	2.86-26.62	38.13	32.56-43.71		
NLR	<2.31	118	49.52	43.23-55.82	0.008	55.31	49.72-60.90	0.007
	≥2.31	87	35.41	29.10-41.72	43.26	36.77-49.74		
PVTT	No	164	49.36	44.04-54.69	p<0.001	55.41	50.69-60.13	p<0.001
	Yes	41	24.60	17.08-32.12	29.18	21.87-36.50		
Recurrence	No					45.37	39.56-51.18	0.026
	Yes					56.71	50.59-62.83	

*CI, confidence interval; HBsAg, hepatitis B surface antigen; AFP, alpha-fetoprotein; BCLC,barcelona-clinic liver cancer; TNM, tumor-node-metastasis; PVTT, portal vein tumor thrombus; LNM,Lymph node metastasis; NLR,neutrophil to lymphocyte ratio

Table 4. Cox Multivariate Proportional Hazard Model of Independent Predictors on Disease-free and Overall Survival

Variable		Hazard ratio (95%CI)	P value
Disease-free survival	AFP ng/ml (≥100 *vs* <100)	1.339 (0.911-1.968)	0.137
	Tumor size, cm (≥4 *vs* <4)	2.918 (1.648-5.166)	p<0.001
	Tumor number (multiple *vs* single)	1.003 (0.614-1.639)	0.989
	BCLC stage (B-C *vs* 0-A)	1.564 (0.878-2.784)	0.129
	PVTT (yes *vs* no)	1.772 (1.100-2.854)	0.019
	NLR (≥2.31 *vs* <2.31)	1.275 (0.878-1.850)	0.202
	DEPDC1 expression (high *vs* low)	1.583 (1.01-2.483)	0.045
Overall survival	AFP ng/ml (≥100 *vs* <100)	1.298 (0.880-1.914)	0.188
	Tumor size, cm (≥4 *vs* <4)	2.592 (1.463-4.589)	0.001
	Tumor number (multiple *vs* single)	1.085 (0.662-1.776)	0.747
	BCLC stage (B-C *vs* 0-A)	1.822 (1.023-3.246)	0.042
	PVTT (yes *vs* no)	1.739 (1.078-2.803)	0.023
	NLR (≥2.31 *vs* <2.31)	1.290 (0.888-1.875)	0.181
	DEPDC1 expression (high *vs* low)	1.651 (1.041-2.617)	0.033

*CI: confidence interval; PVTT: portal vein tumor thrombus; BCLC: barcelona-clinic liver cancer; AFP: alpha-fetoprotein; NLR: neutrophil to lymphocyte ratio

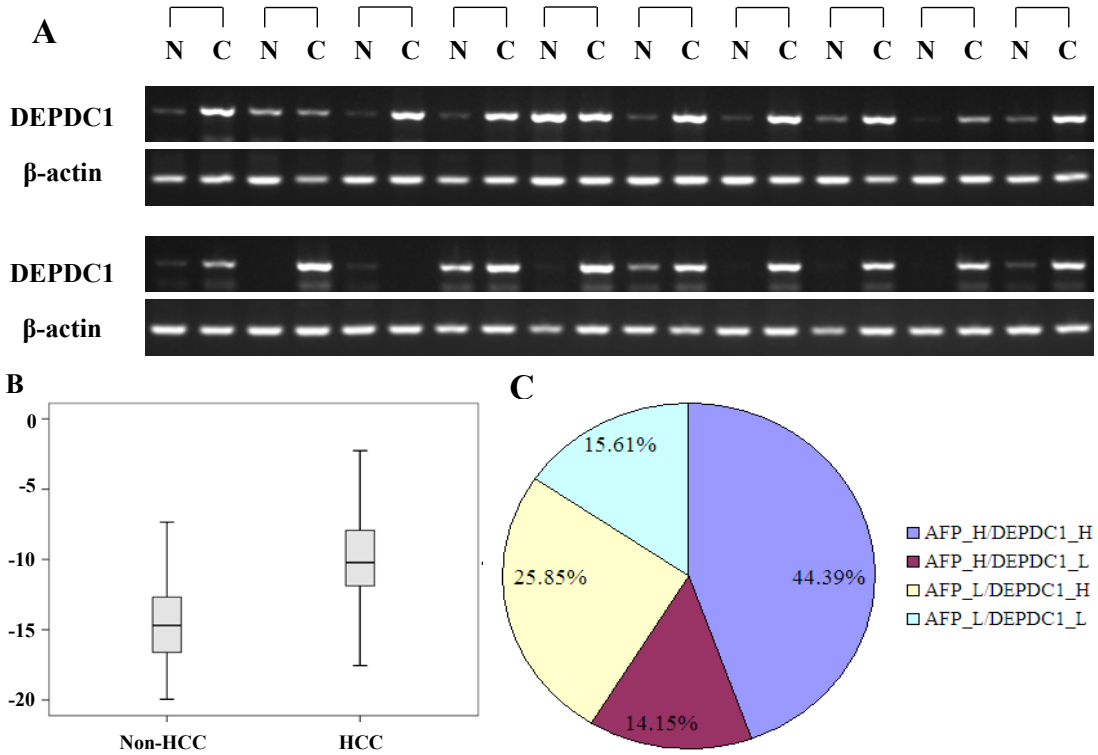

Figure 1. DEPDC1 expression in HCC tissues. A) DEPDC1 expression in 20 cases of HCC tissues (marked as "C" in panel A) and paired adjacent normal liver tissues (marked as "N" in panel A) by semi-quantitative RT-PCR. **B)** Relative DEPDC1 mRNA expression in 205 cases of HCC tissues and adjacent normal liver tissues analyzed by quantitative real-time PCR. Data shown as mean -ΔCT; DEPDC1 mRNA expression in HCC cancer tissues was significantly higher than non-HCC tissues (-9.96±0.2254 *vs* -14.50±0.2104; $p<0.001$). **C)** The distribution of DEPDC1 mRNA (cutoff value: $2^{\Delta CT}\geq 1$) and serum AFP level (cutoff value: AFP ≥100 ng/ml) in 205 HCC patients. The numbers in the pie indicated the percentages of DEPDC1 and/or AFP higher (H) or lower (L) than the cut-off value. (D) DEPDC1 expression in HCC was confirmed by immunohistochemical staining. Representative pictures were shown

increased in 39 cases. AFP and DEPDC1 mRNA both were increased in 48 cases. If combining serum AFP level and DEPDC1 mRNA in liver tissue, the diagnostic rate of HCC could reach more than 80% (Figure 1C).

Correlation between DEPDC1 expression in HCC and clinicopathological parameters

To explore whether increased DEPDC1 expression was relevant to clinicopathological parameters, we performed Chi-square test. As shown in Table 2, High DEPDC1 expression was significantly correlated with serum AFP level (≥100 μg/ml) (χ^2=4.326, p=0.038), tumor size (≥ 4cm) ($\chi^2$1.203, p=0.022), B-C of BCLC stage (χ^2=6.348, p=0.012) and recurrence (χ^2= 7.072, p=0.008). Nevertheless, no significant correlation was observed between high DEPDC1 expression and age, gender, family history, HBsAg, liver cirrhosis, and PVTT (all p>0.05).

High DEPDC1 expression is an independent predictor for DFS and OS

Kaplan-Meier survival analysis revealed that high DEPDC1 expression is related to poor OS and DFS

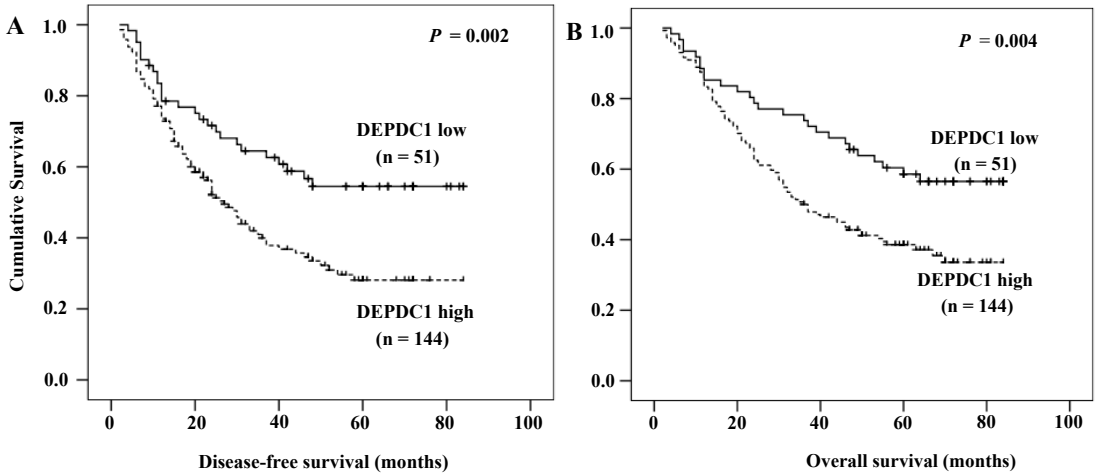

Figure 2. Correlation between DEPDC1 expression and disease free survival (DFS) or overall survival (OS). Patients with high DEPDC1 expression had a shorter DFS A) and OS B). The solid line represents the patient with low DEPDC1 expression, and the dashed line represents the patient with high DEPDC1 expression

(Figure 2). The median OS of patients with DEPDC1 high expression or DEPDC1 low expression were 45.86 [95% confidence interval (95%CI), 40.77-50.95] months and 60.24 (95%CI, 52.74-67.74) months, respectively (p=0.002). The median DFS of patients with DEPDC1 high expression or DEPDC1 low expression were 38.82 (95%CI, 33.36-44.28) months or 55.321 (95%CI, 46.88-63.76) months (p=0.004), respectively. Furthermore, univariate analysis identified size of tumor ≥4cm, B-C of BCLC stage, multiple tumor number, the AFP value (≥100 ng/ml), recurrence together with PVTT and NLR were associated with a shorter DFS and OS (Table 3). Multivariate analysis demonstrated that size of tumor ≥4 cm (HR, 2.918; 95%CI, 1.648-5.166; P <0.001), PVTT (HR, 1.772; 95%CI, 1.100-2.854; p=0.019), high DEPDC1 expression (HR, 1.583; 95%CI, 1.01-2.483; p=0.045) were independent predictors for DFS (Table 4), and that size of tumor ≥4 cm (HR, 2.592; 95%CI, 1.463-4.589; p=0.001), B-C of BCLC stage (HR, 1.882; 95%CI, 1.023-3.246; p=0.042) , PVTT (HR, 1.739; 95%CI, 1.078-2.803; p=0.023) and high DEPDC1 expression (HR, 1.651; 95%CI, 1.041-2.617; p=0.033) were independent predictors for OS (Table 4).

Discussion

In the current study, we detected that DEPDC1 is up-regulated in over 70% HCC tissues compared with adjacent normal liver tissues, found that high DEPDC1 expression is correlated with these clinicopathologic parameters: AFP≥100ng/ml, tumor size ≥4cm, B-C of BCLC stage and recurrence, revealed that high DEPDC1 expression, together with AFP ≥100, tumor size ≥4cm, multiple tumor number, B-C of BCLC stage, PVTT and NLR ≥2.31 were relevant to poor DFS and OS, and that high DEPDC1 expression might be an independent predictor for DFS and OS.

Previous reports have demonstrated that DEPDC1 is up-regulated in bladder cancer (Kanehira et al., 2007), breast cancer (Kretschmer et al., 2011) and lung cancer (Okayama et al., 2012). To our knowledge, our report is the first one showed DEPDC1 up-regulation in HCC. AFP is a broadly used serum HCC marker. But its levels remain normal in up to 40% HCC patients. Thus, it is urgent to identify novel biomarkers to improve diagnostic rate of HCC. Our data demonstrated that HCC positive rate could reach over 80% if serum AFP level and tissue DEPDC1 mRNA are both used as the diagnostic markers. Therefore, DEPDC1 might be a novel diagnostic biomarker of HCC that is capable of compensating the shortcoming of AFP to improve HCC diagnostic rate significantly.

Our study revealed that high DEPDC1 expression, tumor size ≥4cm, PVTT, and B-C of BCLC stage are independent prognostic indicators for DFS and OS. High AFP as an indicator of poor survival was reported previously (Peng et al., 2004; Cheng et al., 2011). A systematic review of 72 studies revealed that PVTT, large tumor size, tumor number are robust indicators of poor prognosis (Tandon and Garcia-Tsao, 2009). Larger size tumor and multifocal tumors tend to invade portal veins, and lead to intrahepatic tumor recurrence and PVTT. Therefore, microvascular portal vein thrombosis is associated with disease free survival and recurrence rates (Ercolani et al., 2003). In addition, cells derived from PVTT showed a typical migratory tendency and aggressive phonotype (Wang et al., 2010). As our data demonstrated that high DEPDC1 expression is associated with these aggressive features, it will be very interesting to elucidate the mechanisms by which DEPDC1 expression is connected to these factors in the future.

High DEPDC1 expression was identified as an independent prognostic indicator for DFS and OS in our study, indicating DEPDC1 may play an important role in tumor development. Given that DEPDC1 is also up-regulated in bladder cancer (Kanehira et al., 2007), breast cancer (Kretschmer et al., 2011) and lung cancer (Okayama et al., 2012), it is crucial to unveil how DEPDC1 is regulated in future study. Researchers found DEPDC1 was up-regulated in bladder cancer tissues, but undetectable in 24 normal human tissues except in testis (Kanehira et al., 2007), and developed peptide vaccine based on this cancer/testis antigen, which effectively

induced peptide-specific cytotoxic T lymphocyte *in vivo* (Obara et al., 2012). Another cell permeable peptide capable of disrupting interaction between DEPDC1 and zinc finger transcription factor ZNF224 showed promising results in bladder cancer cells (Harada et al., 2010). These two reports demonstrated that DEPDC1 is a therapeutic target, at least in bladder cancer. Based on our findings that DEPDC1 is up-regulated in HCC, it might be very interesting to explore the possibility of utilizing DEPDC1 as a therapeutic target in HCC.

In conclusion, DEPDC1 is up-regulated in HCC, and might be a novel HCC diagnostic marker, prognosis predictor, as well as a therapeutic target for HCC patients. Further studies on its regulation and function will ultimately benefit patients with HCC or other related cancers.

List of abbreviations: HCC, hepatocellular carcinoma; DFS, disease-free survival; OS, overall survival; AFP, alpha-fetoprotein; PVTT, portal vein tumor thrombus; NLR, neutrophil to lymphocyte ratio; BCLC, barcelona-clinic liver cancer.

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