Higher expression of A-kinase anchoring-protein 1 predicts poor prognosis in human hepatocellular carcinoma

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Abstract. A-kinase anchoring protein 1 (AKAP1) plays important regulatory roles in the regulation of mitochondrial function, oxidative metabolism, and cell survival. However, the expression pattern and prognostic value of AKAP1 in hepatocellular carcinoma (HCC) remains unclear. AKAP1 expression levels in tumor and matched non-tumor tissues were evaluated using reverse transcription-quantitative polymerase chain reaction and immunohistochemical staining. Kaplan-Meier and Cox regression analyses were used to analyze the survival rates. We found that AKAP1 protein expression was increased in HCC tissues, and high AKAP1 expression was associated with tumor size (P=0.024), Tumor-Node-Metastasis stage (P=0.0296) and portal vein thrombosis (P=0.00498). Kaplan-Meier survival analyses further revealed that high AKAP1 expression was associated with poor overall (P=0.004) and disease-free survival (DFS) (P=0.002) rates in patients with HCC. Multivariate survival analysis revealed that AKAP1 served as an independent poor prognostic factor for DFS rates. The findings of the present study indicated that AKAP1 expression may contribute to HCC progression. High AKAP1 expression could serve as a valuable prognostic biomarker in predicting the survival of patients with HCC following radical resection.

Introduction

As the main histological subtype of liver cancer, hepatocellular carcinoma (HCC) is the third-leading cause of cancer-associated mortality worldwide (1,2). There are >700,000 incidences of morality due to HCC annually (3,4). China in particular has a high incidence rate of HCC, accounting for over half of new cases and mortalities, with >422,100 HCC-associated mortalities in 2015 (5,6). Extensive studies have shown that the long-term prognosis of HCC remains dismal following radical excision owing to the high frequencies of tumor recurrence and distant metastases (6). Therefore, it is necessary to identify novel prognostic factors to guide clinical disease management following surgical resection.

A-kinase anchoring protein 1 (AKAP1, also known as AKAP121 and AKAP149) is a scaffold protein that integrates protein kinase A (PKA) and other signaling components to the outer mitochondrial membrane (7). Recent studies have revealed the roles of AKAP1 in regulating mitochondrial function, oxidative metabolism and cell survival (8,9). AKAP1 recruits the PKA holoenzyme to specific subcellular sites and substrates, which is critical for the physiological actions of the kinase (10). AKAP1 also interacts with protein-tyrosine phosphatase D1 (PTPD1), a non-receptor tyrosine phosphatase that activates Src tyrosine kinase by binding with it (11).

In the heart, AKAP1 deficiency promotes cardiac mitochondrial aberrations and mitophagy, which enhances infarct size, pathological cardiac remodeling and increases the mortality rate from ischemic conditions (9). A more recent study (12) also demonstrated that AKAP1 is a transcriptional target of Myc and supports the growth of cancer cells. Upregulation of AKAP1 in high-grade human tumors is associated with enhanced mechanistic target of rapamycin (mTOR) activation and reduced patient survival. Knockdown of AKAP1 inhibited the mTOR pathway and impaired glioblastoma growth. However, the expression pattern and significance of AKAP1 in HCC remains to be investigated.

Despite the growing evidence of a link between AKAP1 and cancer, little is known about the expression level of AKAP1 and its clinicopathological significance in human HCC. The present study evaluated AKAP1 expression in paired tumorous and non-tumorous HCC samples. Next, the prognostic value of AKAP1 was determined in patients with HCC who previously underwent hepatectomies.

Patients and methods

Patients and samples. Formalin-fixed, paraffin-embedded HCC tissues from 141 male and 17 female patients (age range,

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18 to 78 years; median age, 50 years) who underwent curative resection at Eastern Hepatobiliary Surgery Hospital (Shanghai, China) between September 2006 and July 2011 were randomly retrieved. Another 30 pairs of fresh HCC tissues and the corresponding adjacent non-tumorous tissues (peritumoral tissues) were collected (26 male and 4 female patients; age range, 32 to 72 years; median age, 51 years) for reverse transcription-quantitative polymerase chain reaction (RT-qPCR). All human sample collection procedures were approved by the Biomedical Ethics Committee of Eastern Hepatobiliary Surgery Hospital (Shanghai, China). Informed consent for tissue banking and future medical research was obtained from each participant prior to surgery. The diagnosis of HCC was confirmed by pathological tests in all patients. The clinical staging was determined by the Tumor-Node-Metastasis (TNM) classification system (13). All patients were followed up until December 2014. The overall survival (OS) time was defined as the interval between the dates of surgery and death, and the disease-free survival (DFS) time was defined as the interval between the dates of surgery and first incidence of recurrence. If recurrence was not diagnosed, patients were censored at the date of mortality or the last follow-up.

Public database. A publically available cohort of human HCC patients from the National Center for Biotechnology Information Gene Expression Omnibus (GEO) dataset GSE45436 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE45436) was used. This dataset included data from 39 samples of normal liver tissues and 95 samples of HCC tissues.

RT-qPCR. Total RNA was extracted from the fresh samples using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), following manufacturer instructions. RNA was then reversed transcribed to cDNA with Superscript III RT (Invitrogen; Thermo Fisher Scientific, Inc.) and random primers. Levels of AKAP1 and 18S were measured by SYBR Green qPCR Master Mix (Takara Bio, Inc., Otsu, Japan) using an ABI PRISM7300HT Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The 18S rRNA expression level in the corresponding tissue was used as an internal control. Primers were designed as follows: AKAP1 forward, 5'-GCTTACGGCTTGTACCTG AAG-3' and reverse, 5'-ATGGTGCTCTTGGAAATACGC-3'; and 18S forward, 5'-CGGCTACCACATCCAAGGAA-3' and reverse, 5'-GCTGGAATTACCGCGGCT-3'. The PCR reactions were performed under the following conditions: Initial denaturation at 95˚C for 15 min, followed by 40 cycles of 95˚C for 30 sec, 60˚C for 20 sec and 72˚C for 30 sec. All samples were run in triplicate, and the data were normalized to the 18S internal controls. The relative AKAP1 expression levels were calculated based on the $2^{-\Delta\Delta Cq}$ method (14).

Histopathological and immunohistochemical evaluation. The formalin-fixed paraffin-embedded HCC tissue blocks were cut into 5 μm-thick sections. Then sections were deparaffinized at room temperature by 100% xylene (2 times, 5 min each) and rehydrated using a graded alcohol series (2 changes of 100% ethanol for 5 min each; 95 and 70% ethanol for 5 min each). Following incubation with 3% hydrogen peroxide for 20 min at room temperature, to quench endogenous peroxidase activity, the sections were processed for heat-induced antigen retrieval in sodium citrate buffer (pH=6.0). After several washes with 0.01 M phosphate buffer, the sections were incubated with 10% goat serum (Beyotime Institute of Biotechnology, Shanghai, China) for 30 min at room temperature to block non-specific binding. Next, the slides were incubated with primary anti-AKAP1 antibody (cat no. 5203; dilution, 1:200; Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4˚C. After the primary antibody was washed off, anti-rabbit peroxidase-conjugated secondary antibody (cat no. sc-2357; dilution, 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) incubation was performed for 30 min at room temperature, and then DAB reagent (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) was utilized for detection. The staining of slides was observed and images captured using an Olympus microscope (IX-70 Olympus Corporation, Tokyo, Japan). All sections were evaluated by two independent pathologists who were blind to the clinicopathological data of the patients.

The percentages of positive tumor cells were semi-quantitatively graded as follows: 0, <5%; 1, 5-25%; 2, 26-50%; and 3, >50%. The staining intensity of tumor cells was scored as follows: 0, negative staining; 1, weak staining; 2, moderate staining; and 3, strong staining. The two scores were multiplied to obtain the final immunoreactive score. High expression of AKAP1 in tumor cells was defined as an immunoreactive score ≥4.

Statistical analysis. Each experiment was performed in triplicate and data are presented as the mean ± standard deviation. SPSS version 16 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. χ^2 tests were applied to determine statistical significance. Kaplan-Meier and Cox regression analyses were used to perform survival analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Increased expression of AKAP1 in patients with HCC. To determine the expression of AKAP1 in patients with HCC, RT-qPCR assays were performed for 30 pairs of HCC tissues and the corresponding adjacent non-tumorous tissues (peritumoral tissues). As shown in Fig. 1A, AKAP1 mRNA levels were significantly higher in HCC tissues. Consistently, analysis of public microarray data (GEO accession number GSE45436) also revealed that AKAP1 is significantly upregulated in tumor samples (Fig. 1B). To investigate AKAP1 expression in HCC tissues further, its expression was assessed in 158 cases of HCC and matched adjacent tissues by immunohistochemical (IHC) staining. As shown in Fig. 1C-E, the expression pattern of AKAP1 varies significantly among the HCC samples. The expression of AKAP1 was significantly increased in HCC samples relative to paired non-cancerous tissues in from 64.6% (102/158) of patients with HCC (Fig. 1F).

High AKAP1 expression is associated with aggressive clinicopathological features. Based on IHC staining intensity and percentages of positive tumor cells (Fig. 2), the patients were subdivided into two groups: High (n=107) and low (n=51) AKAP1 expression groups. As depicted in Table I, AKAP1

Table I. Association between AKAP1 expression and clinicopathological characteristics in 158 hepatocellular carcinoma patients.

Variables	All cases	Low AKAP1	High AKAP1	P-value
Total	158	51	107	
Sex				0.414
Female	17	4	13	
Male	141	47	94	
Age, years				0.847
<50	73	23	50	
≥50	85	28	57	
HBV infection				0.944
Yes	139	45	94	
No	19	6	13	
Liver cirrhosis				0.420
Yes	112	34	78	
No	46	17	29	
AFP, ng/ml				0.828
≥200	91	30	61	
<200	67	21	46	
Tumor multiplicity				0.325
Single	55	15	40	
Multiple	103	36	67	
Tumor size, cm				0.024
≤5	52	23	29	
>5	106	28	78	
Tumor encapsulation				0.395
Absent	79	23	56	
Present	79	28	51	
Edmondson grade				0.903
I/II	35	11	24	
III/IV	123	40	83	
Portal vein thrombosis				0.005
Absence	117	45	72	
Gross	41	6	35	
Pathologic TNM stage				0.030
Early stage (I-II)	92	36	56	
Late stage (III)	66	15	51	

AKAP1, A-kinase anchoring protein 1; HBV, hepatitis B virus; AFP, α-fetoprotein; TNM, Tumor-Node-Metastasis.

expression levels were significantly higher in HCC patients with increased tumor size (P=0.024), portal venous invasion (P=0.00498), and late TNM stage (P=0.0296). These data indicate that high AKAP1 expression is associated with aggressive clinicopathological features.

High AKAP1 expression predicts poor overall survival (OS) and disease-free survival (DFS) rates in a cohort of patients with HCC. To assess the association between AKAP1 expression levels with survival of patients with HCC, Kaplan-Meier survival analyses were performed. As depicted in Fig. 3, patients in the high expression group exhibited poorer DFS (P=0.002) and OS (P=0.004) rates than those in the low expression group. In addition, to determine whether AKAP1 expression level is an independent prognostic factor for DFS rate and identify other prognostic factors for DFS rate in patients with HCC who underwent curative resection, Cox regression analysis was performed for 12 clinicopathological variables. Univariate analysis demonstrated that AKAP1 expression level was associated with DFS rate [hazard ratio (HR), 1.934; 95% confidence interval (CI), 1.243-3.01; P=0.003], and our multivariable Cox regression analyses further confirmed that AKAP1 expression levels were an independent risk factor of DFS rate (HR, 1.972; 95% CI, 1.177–3.306; P=0.01) (Table II). These data indicated that high AKAP1 expression in tumors was

Table II. Univariate and multivariate Cox regression analysis of risk factors for disease-free survival rate.

	Univariate analysis		Multivariable analysis	
Variables	Hazard ratio (95% CI)	P-value	Hazard ratio (95% CI)	P-value
Sex (male vs. female)	0.512 (0.249-1.055)	0.070		
Age (≥50 vs. <50 years)	0.876 (0.595-1.289)	0.501		
HBV infection (present vs. absent.)	1.067 (0.585-1.948)	0.832		
Liver cirrhosis (present vs. absent)	1.737 (1.083-2.787)	0.022	1.681 (0.999-2.829)	0.051
AFP (≥400 ng/ml vs. <400 ng/ml)	1.245 (0.842-1.841)	0.272		
Tumor multiplicity (multiple vs. single)	1.593 (1.073-2.365)	0.021	0.963 (0.592-1.568)	0.880
Maximal tumor size (≥5 vs. <5 cm)	2.033 (1.303-3.174)	0.002	2.110 (1.275-3.493)	0.004
Tumor encapsulation (absent vs. present)	1.707 (1.111-2.621)	0.015	1.381 (0.865-2.206)	0.176
Edmondson grade (III/IV vs. I/II)	1.631 (0.981-2.71)	0.059		
Portal vein thrombosis (gross vs. absence)	2.329 (1.543-3.516)	<0.001	1.461 (0.892-2.395)	0.132
Pathologic TNM stage (III vs. I-II)	1.800 (1.225-2.645)	0.003	1.354 (0.861-2.130)	0.189
AKAP1 level (high vs. low)	1.934 (1.243-3.01)	0.003	1.972 (1.177-3.306)	0.010

AKAP1, A-kinase anchoring protein 1; HBV, hepatitis B virus; AFP, α-fetoprotein; TNM, Tumor-Node-Metastasis; CI, confidence interval.

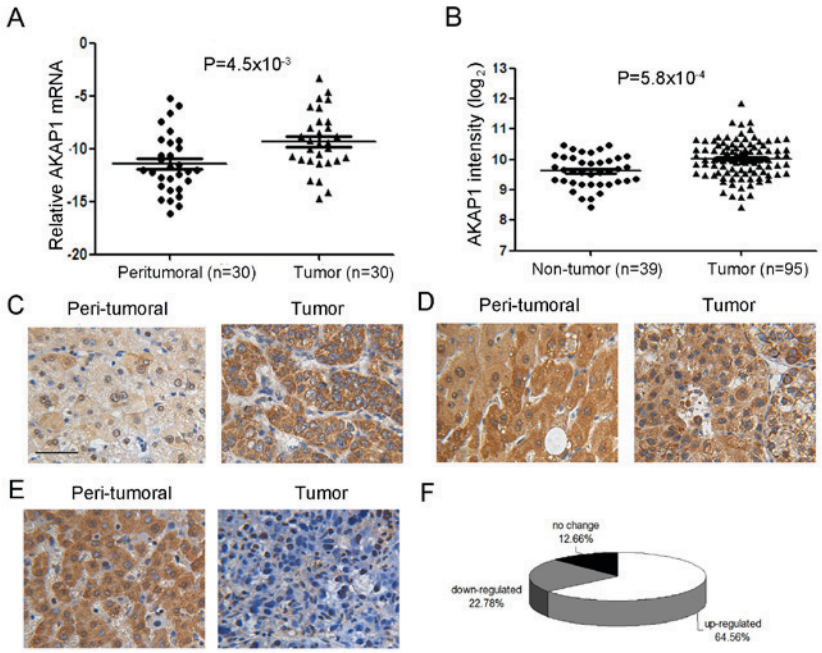

Figure 1. AKAP1 is frequently upregulated in HCC tissues. (A) The mRNA expression of *AKAP1* was examined by reverse transcription-quantitative polymerase chain reaction in 30 pairs of HCC tissues and matched peritumoral tissues. (B) AKAP1 expression was analyzed in HCC tumor and non-tumor tissues that were obtained from the Gene Expression Omnibus (accession number GSE45436). (C-E) Representative immunohistochemical staining of AKAP1 in tumor tissues and matched peritumoral tissues. (C) Upregulated (D) no change, and (E) downregulated. Scale bar, 50 μm. (F) The expression of AKAP1 was upregulated in 64.56% (102/158) of patients with HCC. AKAP1, A-kinase anchoring protein 1.

associated with disease recurrence and poor survival rates for patients with HCC, and AKAP1 could also be used as a valuable prognostic factor for the DFS rate of patients with HCC who underwent curative resection.

Discussion

Recently, evidence indicates that metabolic disorders have notable roles in HCC initiation, progression and therapy resistance. Obesity and diabetes have long been recognized as independent risk factors for the development of HCC (15-17).

As the energetic hub of the cell, mitochondria serve important roles in regulating cell function and survival (18,19). Mitochondrial dysfunction is a common trait of several human diseases, including cancer (20-23). AKAP1 is a scaffolding protein that functions as the key regulatory molecule responsible for controlling mitochondria function. Therefore, it is

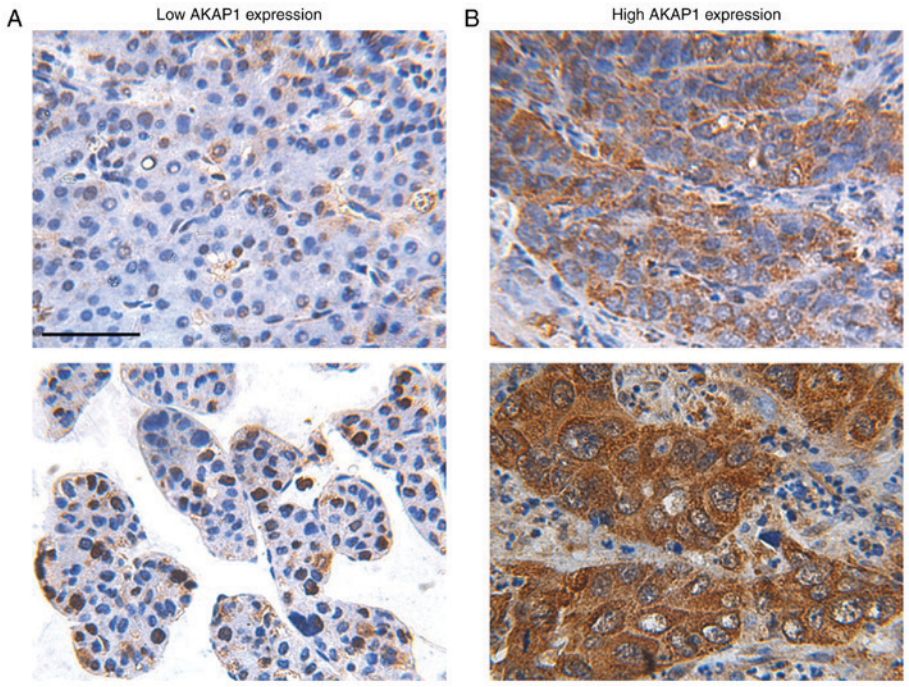

Figure 2. Representative immunostaining of AKAP1 in HCC. AKAP1 expression was evaluated by immunohistochemically staining. Representative (A) low and (B) high AKAP1 expression samples were shown. Scale bar, 50 μm. AKAP1, A-kinase anchoring protein 1.

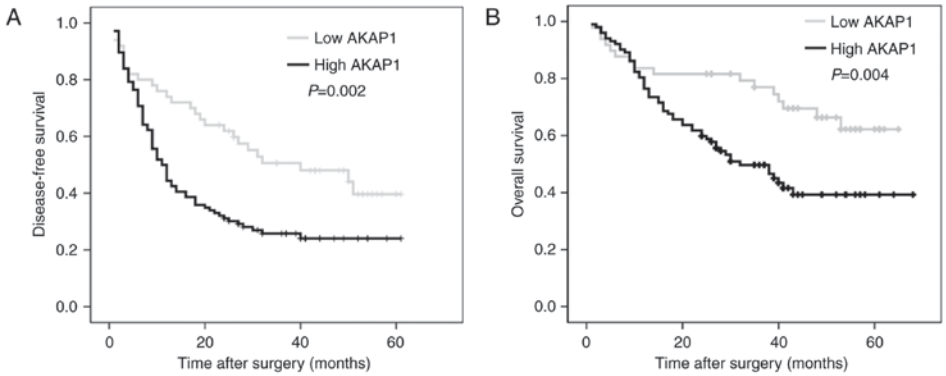

Figure 3. High expression of AKAP1 in tumors is associated with poor survival of patients with HCC. The (A) disease-free and (B) overall survival rates of 158 patients with HCC were compared between the low- and high-AKAP1 expression groups. AKAP1, A-kinase anchoring protein 1; HCC, hepatocellular carcinoma.

necessary to investigate the role of AKAP1 in cancer. In fact, the expression profiles and prognostic implications of AKAP1 have been documented in human non-small cell lung cancer, and AKAP1 overexpression is inversely associated with patient survival (13). The present study evaluated the expression level of AKAP1 in HCC for the first time. Through studying human HCC clinical specimens, it was revealed that AKAP1 was overexpressed in the majority of patients with HCC, and high AKAP1 expression was associated with aggressive clinicopathological features. These data indicated that AKAP1 may contribute to progression of HCC.

Although early diagnosis and developments in surgery improved the short-term survival rates of patients with HCC (24), the long-term prognosis of patients with HCC remains unsatisfactory, even following radical excision. Therefore, it is necessary to reveal reliable biomarkers for the effective identification of patients with HCC who have a high risk of relapse following surgery. In the present study, Kaplan-Meier survival analyses revealed that high AKAP1 expression was associated with poor OS and DFS rates. Furthermore, univariate and multivariate survival analyses indicated that AKAP1 expression could serve as an independent factor for the DFS rate of patients with HCC following hepatectomy.

In conclusion, the results of the present study demonstrated that AKAP1 was overexpressed in the majority of patients with HCC, and its increased expression was associated with poor patient prognosis. AKAP1 may serve as a valuable prognostic biomarker in predicting the survival of patients with HCC following radical resection.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JY and RL designed the experiments. JY, YZ, DXZ and JW performed the experiments, analyzed and interpreted the data, and wrote the manuscript. RL organized and supervised the study. All authors read and approved the final manuscript.

Ethics approval and consent to publish

All human sample collection procedures were approved by the Biomedical Ethics Committee of Eastern Hepatobiliary Surgery Hospital (Shanghai, China). Written informed consent for tissue banking and future medical research was obtained from each participant prior to surgery.

Consent for publication

Identifying information of patients were not included in the manuscript. Informed consent for tissue banking and future medical research was obtained from each participant prior to surgery.

Competing interests

The authors declare that they have no competing interests.

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