RESEARCH ARTICLE

Differential Expression of IQGAP1/2 in Hepatocellular Carcinoma and its Relationship with Clinical Outcomes

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Abstract

Purpose: To investigate IQGAP1 and IQGAP2 expression in hepatocellular carcinoma (HCC) and itsassociation with HCC clinicopathological characteristics and survival outcomes. Methods: IQGAP1 and IQGAP2 mRNA and protein were measured in HCC tissues, para-tumor tissues and normal tissues by RT-PCR and Western blotting. We further examined 150 HCC samples with adjacent para-tumor tissues and 11 normal specimens by immunohistochemistry to evaluate the correlation of IQGAP1 and IQGAP2 with clinicopathological features and prognosis. Results: IQGAP1 mRNA and protein were up-regulated while IQGAP2 mRNA and protein were down-regulated in human HCC tissues compared with para-tumor and normal liver tissues ($p<0.05$). IQGAP1 expression was higher in primary HCC (122/150, 81.3%) than matched adjacent tissues (30/150, 20%, $p<0.001$), whereas IQGAP2 was lower (31/150, 20.7% as compared to 112/150, 74.7%, P<0.001). Positive IQGAP1 expression correlated with larger tumor size ($p=0.002$), advanced TNM stage ($p=0.002$) and tumor differentiation (III and IV, $p=0.034$). Negative IQGAP2 expression was significantly associated with larger tumor size ($p=0.009$), multicentric tumor occurrence ($p=0.01$), advanced TNM stage (0.009) and tumor differentiation (III and IV, $p=0.020$). Survival analysis revealed that patients with either IQGAP1+ or IQGAP2- tumors had significantly reduced disease-free survival ($p<0.001$ and 0.006 respectively) and overall survival ($p<0.001$ for both). Multivariate analysis showed that IQGAP1/2 switch was an independent prognosis factor for disease-free survival (HR=2.824) and overall survival (HR=2.189). Conclusion: Positive IQGAP1 and negative IQGAP2 expression were closely correlated with tumor progression and could be used as adjunctive biomarkers to improve prognostication for HCC patients.

Keywords: IQGAP1 - IQGAP2 - hepatocellular carcinoma - survival analysis

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Introduction

Hepatocellular carcinoma (HCC) is a major form of liver cancer and has become the fifth most common malignancy and the third leading cause of cancer related mortality worldwide (Gomaa et al., 2008). Predisposing factors for HCC include chronic hepatitis B and C virus infections, exposure to aflatoxin B1, chronic alcohol consumption, and any hepatic diseases associated with cirrhosis (Thomas, 2009). The current treatments for HCC with established efficacy include: surgical resection/liver transplantation, transcatherter arterial chemoembolization, percutaneous radiofrequency ablation, percutaneous ethanol injection, percutaneous microwave coagulation therapy and molecular targeted therapy (e.g. sorafenib) (Maluccio et al., 2012; Berk et al., 2013; Nishikawa et al., 2013). Although the clinical outcomes of HCC have significantly improved, the survive rate remains poor due to the high rate of recurrence. Therefore, it is crucial to better understand carcinogenesis of HCC and seek new biomarkers for potential targeted therapy.

IQ-motif containing GTPase-activating proteins (IQGAPs) represents a small subgroup of evolutionally conserved superfamily of GTPase-activating proteins (Bernards, 2003). Mammalian cells contain three IQGAPs, IQGAP1, IQGAP2 and IQGAP3. They share a high degree of sequence homology and a similar domain structure, and differ in tissue distribution (White et al., 2009). All three are large cytoplasmic scaffolding proteins (MW 180-190 kDa). Their domain structure includes an actin binding calponin homology (CH) domain, a single WW domain capable of binding various proline-rich proteins, four IQ motifs binding calmodulin, a large GTPase binding domain (GBD) known to bind Rho GTPases Rac1 and cdc42, and a RasGAP C-terminus domain (RGCT) (Brown et al., 2006). IQGAP1 is the best characterized one, and its binding partners involve in several cell signal pathways. The function of IQGAP1 protein includes cytoskeltal regulation, coordinating cadeherin mediated cell-cell adhesion, cell polarization and actin reorganization to

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promote cell migration. Accumulating evidence strongly supports IQGAP1 is an oncogene (Casteel et al., 2012; White et al., 2012). A comparison of structure and function of IQGAP2 with those of IQGAP1 reveals some similarities and several differences. The human IQGAP2 protein exhibits 59% identity to IQGAP1, and their domain structure are well conserved (Briggs et al., 2003). Binding to diverse partners, IQGAP2 and IQGAP1 may play opposing roles in carcinogenesis (Jin et al., 2008; Schmidt et al., 2008; Schmidt, 2012).

It has been demonstrated that IQGAP1 and IQGAP2 are reciprocally altered in HCC (Schmidt et al., 2008; White et al., 2010). The increased IQGAP1 and/or decreased IQGAP2 expression may contribute to the pathogenesis of human HCC. However, the prognostic implication of IQGAP1/2 in HCC remained uncertain. In this study, we investigated the expression patterns of IQGAP1/2 in clinical HCC tissues and evaluated their prognostic roles in 150 HCC patients after curative hepatectomy with long-term follow-up and extensive information on clinicopathologic characteristics.

Materials and Methods

Patient selection

Fresh tissues were collected from 21 patients with HCC who underwent curative hepatectomy (defined as tumor tissues and para-tumor tissues) and 11 patients who experienced operation due to parenchyma of hepatic hemangioma (normal liver tissues acquired from the incisal edge, defined as normal control tissues) in the department of General Surgery, Xiangya Hospital, Central South University between Mar 2013 and Aug 2013. These 32 tissues were used to detect the mRNA and protein levels of IQGAP1 and IQGAP2. The study was approved by the Institutional Review Board at Xiangya Hospital, Central South University, Changsha, China.

A consecutive series of 150 previously untreated patients who received curative hepatectomy in our department between Jan 2007 to Dec 2007 were enrolled. All patients were histopathological diagnosed and followed-up. In this series, adjacent para-tumor tissues and 11 embedded normal tissues were also included. Their paraffin blocks were used for Immunohistochemistry assay. Curative resection was defined according to BCLC staging system, and patients with a major portal vein tumor thrombus and tumor rupture certified before or during operation were excluded (Bruix et al., 2011). Patients with serious diseases and other malignant tumors were also excluded. The patients' characteristics were shown in Table 1. Tumor stage was defined according to 7th edition tumor-node-metastasis (TNM) classification of American Joint Committee on cancer (AJCC), and tumor differentiation was assessed according to Edmonson and Steiner grading system.

Real-time reverse transcription polymerase chain reaction (RT-PCR)

The relative cDNA level of IQGAP1/2 in fresh tumor samples were measured by quantitative real-time RT-PCR using ABI 7900 PCR Detection System with SYBR green dye (ABI). Primer sequence for IQGAP1 is: forward: 5'-AGAACGTGGCTTATGAGTACCT-3'; reverse: 5'-CCAGTCGCCTTGTATCTGGT-3'. Primer sequence for IQGAP2 is: forward 5'-TGTGCCACTTAGAGGAAGCC-3', reverse: 5'-GCTCTTAACCACTGGACGGTAT-3'. The relative quantification of IQGAP1/2 cDNA level was compared with internal control gene GAPDH. All samples were run in triplicate.

Western blot analysis

Briefly, total proteins were separated on 15% SDS-PAGE gel then transferred to immune-blot PVDF membrane for immunoblot analysis. The membranes were blocked with 5% deffated milk before exposing to mouse monoclonal anti-IQGAP1 antibody against human IQGAP1 and anti-IQGAP2 antibody against human IQGAP2 (ABCAM, Cambridge, MA, USA; dilution 1:1000). Secondary antibody used in this study was goat anti-mouse antibody with a horseradish peroxidase-conjugated. The experiments were performed for at least 3 times.

Immunohistochemistry (IHC)

Immunohistochemical staining was done on formalin-fixed and paraffin-embeded tissue using 5-μm section from tissue microarray blocks. Mounted tissue section were baked at 60°C for 30 min, deparaffinized in xylene and rehydrated through graded alcohols. Antigen was retrieved by heating in 1μM sodium citrate (PH 6.0) in a pressure cooker for 2 min. According to the manufacturer's instruction, anti-IQGAP1 (dilution 1:500) and anti-IQGAP2 antibody (dilution 1:100) were incubated on the section overnight at 4°C after non-specific staining was blocked, followed by incubation with a horseradish peroxidase conjugated secondary antibody. Staining was visualized using DAB chromogen substrate and counterstained with haematoxylin.

IHC evaluation

All stained sections were assessed independently by two pathologists without knowledge of clinicopathologic features, and any differences in interpretation were resolved by consulting a third pathologist to achieve consensus. Staining for each antibody was considered positive if more than 10% of cells stained strongly in the cytoplasm as described(White et al., 2010; Eom et al., 2011).

Follow up

All the enrolled 150 patients were followed up for 5 years. The disease-free survival was defined as the length of time after hepatectomy for HCC during which a patient survived with no signs of HCC recurrence. Overall survival was defined as the interval between the time of hepatectomy to either death or the last date of follow-up.

Statistical analysis

Group differences were compared with the Student's t-test and Chi-squared test or Fisher's exact test. A spearman's rank correlation was performed to analyze the correlation. Univariate survival analysis was done

Table 1. Correlation Between the Clinicopathologic Variables and IQGAP1/2 Expression in HCC

variables	n	IQGAP1		*p*-value	IQGAP2		*p*-value
		positive	negative		positive	negative	
Age							
≤55	102	84	18	0.389	21	81	0.566
>55	48	38	10		10	38	
Gender							
male	123	99	24	0.398	26	97	0.497
female	27	23	4		5	22	
HBV infection							
absent	14	10	4	0.25	5	27	0.299
present	136	112	24		26	92	
Liver cirrhosis							
absent	101	84	17	0.27	19	82	0.274
Present	49	38	11		12	37	
AFP level							
≤400 ng/ml	118	94	24	0.23	23	95	0.323
>400 n/ml	32	28	4		8	24	
Child-pugh							
A	144	117	27	0.689	31	113	0.243
B	6	5	1		0	6	
Tumor size							
≤5cm	57	39	18	0.002	18	39	0.009
>5cm	93	83	10		13	80	
Multicentric occurrence							
-	123	97	26	0.076	30	93	0.01
+	27	25	2		1	26	
Tumor encapsulation							
Complete	106	86	20	0.56	22	76	0.488
Incomplete	44	36	8		9	35	
TNM stage							
I and II	57	39	18	0.002	18	39	0.009
III and IV	93	83	10		13	80	
Tumor differentiation							
I and II	126	99	27	0.034	30	96	0.02
III and IV	24	23	1		1	23	

HBV, hepatitis B virus; AFP, alpha fetoprotein

by the Kaplan-Meier method, and multivariate analysis was done by the Cox proportional hazards regression model. All statistical analysis was performed using SPSS 19.0 software. $P<0.05$ was considered to be statistically significant.

Results

Increased IQGAP1 expression and decreased IQGAP2 expression in HCC

IQGAP1/2 mRNA and protein expression were detected and analyzed in all fresh tissues from the 32 patients. The results showed that IQGAP1 mRNA and protein expression level were significantly higher in tumor tissues than para-tumor ($p<0.001$ and 0.002 respectively) and normal tissues ($p<0.001$ and 0.030 respectively, Figure 1 and 2). On the contrary, IQGAP2 mRNA and protein expression level were significantly lower in tumor tissues than para-tumor ($p<0.001$ and 0.007 respectively) and normal tissues ($p=0.001$ and 0.004 respectively, Figure 1 and 2).

The relationship between clincopathological variables and IQGAP1/2 expression

To further identify the relationship of IQGAP1/2 expression with clincopathological variables of HCC, detections of IQGAP1/2 expression using Immunohistochemistry were carried out in 150 paraffin-embeded tumor tissues with adjacent para-tumor tissues and 11 normal tissues (Figure 3). IQGAP1 expression level was higher in primary HCC (122/150, 81.3%, Figure 3a) than in matched adjacent tissues (30/150, 20%, Figure 3b, $p<0.001$), whereas IQGAP2 was lower in primary HCC (31/150, 20.7%, Figure 3d) than in matched adjacent tissues (112/150, 74.7%, Figure 3e, $P<0.001$). The expressions of IQGAP1 and IQGAP2 were positive in 2/11 (18.2%) and 9/11 (81.8%) normal tissues (Figure 3c and 3f). These results were accordant with the results from RT-PCR and Western blot.

The results demonstrated that positive IQGAP1 expression was correlated with larger tumor size, advanced TNM stage and tumor differentiation (III and IV) (Table 1). Negative IQGAP2 expression was significantly associated with larger tumor size, muticentirc tumor occurrence, advanced TNM stage and tumor differentiation (III and IV) (Table 1). Moreover, spearman`s rank analysis showed that IQGAP1 expression was negatively correlated with IQGAP2 expression ($p<0.001$).

Correlation of IQGAP1/2 protein expression with survivals

Survival data were available for all 150 post-surgical

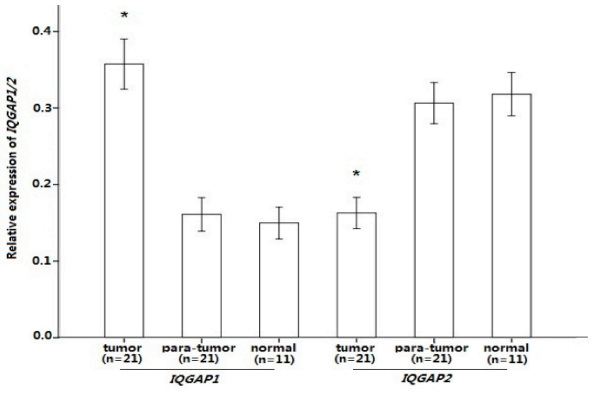

Figure 1. Relative IQGAP1/2 mRNA Value of HCC, Para-tumor and Normal Tissues. Up-regulation of IQGAP1 and down-regulation of IQGAP2 in HCC tumor compared to para-tumor and normal liver tissues. (* $p<0.05$)

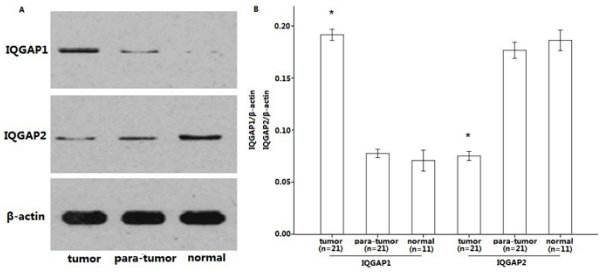

Figure 2. Differential Expressions of IQGAP1/2 Proteins in Tumor, Para-tumor and Normal Tissues. A: representative results of Western blotting analysis for IQGAP1 and IQGAP2 expression. B: IQGAP1 and IQGAP2 protein expression. The expression of IQGAP1 and IQGAP2 protein was up-regulated and down-regulated in HCC tumor in comparison with para-tumor and normal liver tissues (*$p<0.05$)

Table 2. Univariate Analysis of Disease-free Survival and Overall Survival in the 150 HCC Patients

Variables	Disease-free Survival		Over-all Survival	
	HR (95% CI)	*p*	HR (95% CI)	*p*
Age	0.793 (0.521-1.207)	0.279	0.696 (0.422-1.147)	0.155
Gender	0.738 (0.431-1.181)	0.205	0.782 (0.452-1.355)	0.381
HBV infection	1.147 (0.579-2.273)	0.693	1.273 (0.554-2.928)	0.57
Liver cirrhosis	1.007 (0.671-1.512)	0.972	0.985 (0.616-1.575)	0.985
AFP level	0.970 (0.606-1.552)	0.899	1.095 (0.647-1.852)	0.736
Child-pugh	0.968 (0.356-2.631)	0.95	0.984 (0.310-3.121)	0.979
Tumor size	6.228 (3.692-10.509)	<0.001	4.745 (2.612-8.624)	<0.001
Multicentric occurrence	2.150 (1.350-3.423)	0.001	2.583 (1.550-4.305)	<0.001
Tumor encapsulation	1.527 (1.013-2.304)	0.043	1.981 (1.258-3.119)	0.003
TNM stage	1.789 (1.183-2.706)	0.006	1.845 (1.135-3.000)	0.014
Tumor differentiation	6.995 (4.175-11.718)	<0.001	7.451 (4.428-12.537)	<0.001
IQGAP1	3.356 (1.748-6.542)	<0.001	2.817 (1.359-5.848)	0.006
IQGAP2	4.831 (2.433-9.615)	<0.001	4.082 (2.008-10.638)	<0.001

HR, hazard ratio; CI, confidence interval

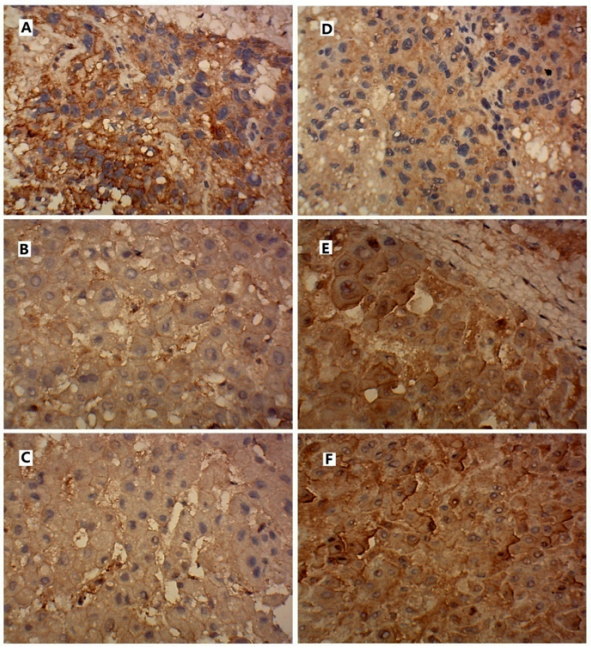

Figure 3. Immunohistochemistry Staining of IQGAP1/2 in HCC, Para-tumor and Normal Tissues. A, positive expression of IQGAP1 in tumor tissue. B, negative expression of IQGAP1 in para-tumor tissue. C, negative expression of IQGAP1 in normal tissue. D, negative expression of IQGAP2 in tumor tissue. E, positive expression of IQGAP2 in para-tumor tissue. F, positive expression of IQGAP2 in normal tissue. (Original magnification: ×400)

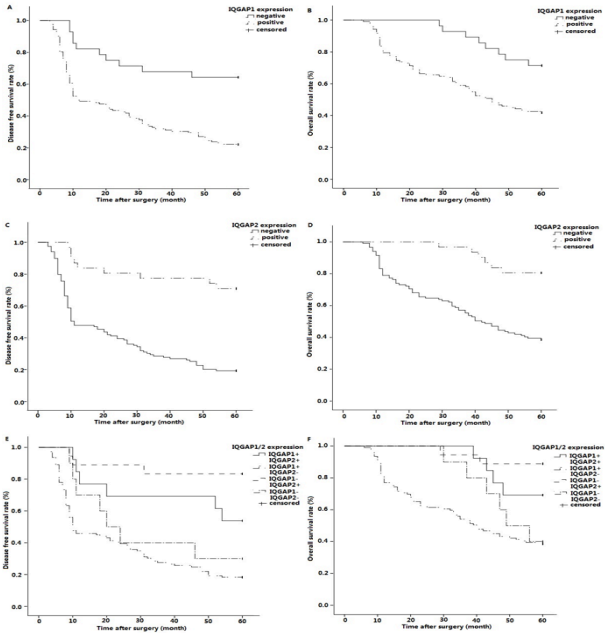

Figure 4. Kaplan-Meier Disease-free Survival and Overall Survival Analysis of IQGAP1 and IQGAP2 Expression in HCC Patients. Left and right column represent disease-free survival and overall survival respectively. A and B for IQGAP1. C and D for IQGAP2. E and F for different IQGAP1/2 expression patterns. Patients with either IQGAP1+ or IQGAP2- tumors had significantly reduced disease-free survival and overall survival. IQGAP1+/IQGAP2- group had the worst outcome while IQGAP1-/IQGAP2+ group had the best outcome for both disease-free survival and overall survival rate

HCC patients. The 1, 3, 5- year disease-free survival rate were 55%, 38.6%, 30% respectively. Univariate analysis showed that larger tumor size, mutilcentric tumor occurrence, incomplete tumor encapsulation, advanced TNM stage, tumor differentiation (III and IV) degree, positive IQGAP1 expression and negative IQGAP2 expression were prognostic factors for disease free survival (Table 2). The 1, 3, 5- year overall survival of the whole group was 83.3%, 65.1%, 48% respectively. Univariate analysis showed that larger tumor size, mutilcentric tumor occurrence, incomplete tumor encapsulation, advanced TNM stage, tumor differentiation (III and IV) degree, positive IQGAP1 expression and negative IQGAP2 expression were prognostic factors for overall survival (Table 2). Patients with IQGAP1+ or IQGAP2- tumors had significantly reduced disease-free survival (p<0.001 and p=0.006 respectively, Figure 4a and 4b) and overall survival (p<0.001 for both, Figure 4c and 4d).

Subsequently, we analyzed the survival curves of 4 groups according to the expressional profile of IQGAP1 and IQGAP2. Patients with IQGAP1+/IQGAP2- tumor had the poorest survival, while the IQGAP-/IQGAP2+ group had the most favorable survival (Figure 4e and 4f). No significant difference were observed between the other two groups. Multivariate analysis indicated that the positive IQGAP1 expression with negative IQGAP2 expression (IQGAP1+/IQGAP2- expression pattern,

Table 3. Multivariate Analysis of Disease-free Survival and Overall Survival for HCC after Surgery

variables	Disease-free Survival		Over-all Survival	
	HR (95% CI)	*p*	HR (95% CI)	*p*
Multicentric occurrence	2.075 (1.294-3.328)	0.002	2.557 (1.511-4.327)	0.016
Tumor encapsulation	1.781 (1.169-2.711)	0.007	2.314 (1.453-3.684)	<0.001
TNM stage	1.827 (1.197-2.789)	0.005	2.086 (1.261-3.449)	0.004
Tumor encapsulation	6.232 (3.698-1.504)	<0.001	6.897 (4.056-11.728)	<0.001
IQGAP1/2 switch	2.824 (1.628-4.896)	<0.001	2.189 (1.160-4.130)	0.016

IQGAP1/2 switch designated as IQGAP1+ and IQGAP2- (compared with other three expression patterns: IQGAP1+/IQGAP2+, IQGAP1-/IQGAP2+ and IQGAP1-/ IQGAP2-)

designated as IQGAP1/2 switch) was an independent prognosis factor for disease-free survival (Hazard Ratio 2.824) and overall survival (Hazard Ratio 2.189) as well as multicentric occurrence, incomplete tumor encapsulation, advanced TNM stage and tumor differentiation (III and IV) (Table3).

Discussion

Recent studies have demonstrated IQGAPs involved in tumorigenesis. IQGAP1 is overexpressed in colorectal carcinoma (Nabeshima et al., 2002), breast cancer (Jadeski et al., 2008), aggressive ovarian adenocarcinomas (Dong et al., 2008) and gastric cancer (Walch et al., 2008), while IQGAP2 expression is lost in gastric carcinoma (Jin et al., 2008), prostate (Xie et al., 2012) and liver cancer (Schmidt, 2012; Gnatenko et al., 2013). It was also reported that IQGAP1 expression is up-regulated in HCC, characterized by the loss of membrane E-cadherin expression, the cytoplasmic translocation of β- catenin, and the overexpression of nuclear target of β- catenin, cyclin D1 (Chen et al., 2010). What's more, tumor suppressor gene Iagap2 is linked to the development of HCC and the activation of Wnt/β- catenin signaling pathway. Inactivation of IQGAP1 in mouse liver impairs tumorigensis caused by IQGAP2 deficiency (Schmidt et al., 2008; Gnatenko et al., 2013). In present study, we demonstrated that IQGAP1 mRNA and protein were up-regulated and IQGAP2 mRNA and protein were down-regulated in human HCC tissues, compared to those in para-tumor and normal liver tissues. These findings suggested that IQGAP1 and IQGAP2 may possess opposing function in the pathogenesis of HCC, and were in accordance with the data observed by other investigators (White et al., 2010).

IQGAP1/2 sharing the same domain structure and significant homology, however, involve in different signaling pathways regulating cell proliferation, transformation, cell motility and invasion (White et al., 2009; Schmidt, 2012). In our study, the expression profiles of IQGAP1 and IQGAP2 were analyzed in a large series of human HCC with direct correlation to clinical and survival data. We demonstrated that positive IQGAP1 and negative IQGAP2 expression were related to some unfavorable clinicpatologic parameters which are associated with advanced tumor stages including larger tumor size, muticentirc tumor occurrence, advanced TNM stage, incomplete tumor encapsulation and tumor differentiation (III and IV). Moreover, positive IQGAP1 expression was significantly associated with negative IQGAP2 expression. Our findings strongly suggested that positive IQGAP1 and negative IQGAP2 were significantly correlated with HCC progression, and provided the evidence that IQGAP1 and IQGAP2 may have distinct functions. Accumulating evidences have elucidated the mechanisms of how IQGAP2 counteracts the effect of IQGAP1 in cancer. Although both IQGAP1 and IQGAP2 binding Rac1 and cdc 42, it appears that IQGAP1 selectively binds to an inacvtive GDP bound form of these GDPases, while IQGAP2 binds both GDP- and GTP-bound forms (Brill et al., 1996; Hart et al., 1996; McCallum et al., 1996; Joyal et al., 1997; Schmidt, 2012). This is particularly relevant to HCC, because targeted ablation of cdc42 in mouse hepatocytes and bile ducts resulted in the developed of HCC (Van Hengel et al., 2008; Schmidt, 2012). IQGAP1 and IQGAP2 are phosphorylated at ser1443 by protein kinase Cε (PKCε) and Thr716 by cAMP-dependent protein kinase (PKA) respectively (Breuhahn et al., 2006; Elliott et al., 2012). PKCε and PKA kinases regulate distinct signaling pathway, regarding cancer progression, and their dissimilar activators and targets may also hold a key to deciphering the mechanisms (Grohmanova et al., 2004; Wang et al., 2009; Schmidt, 2012;).

HCC is a heterogeneous cancer with high mortality. Searching for valuable biomarkers for HCC diagnosis and prognostic prediction has been attracting an increasing number of experts. Plenty of proteins have been shown to have clinical significance for predicting HCC prognosis (Luo et al., 2013; Wang et al., 2014; Yu, 2014). The availability of these clinically applicable biomarkers remains limited, thus there is an urgency to strengthen the role of these biomarkers as prognostic factors for HCC patients. A lack of IQGAP1 related with favorable prognosis has been observed in gastric cancer (Walch et al., 2008). Through this retrospective study of the 150 patients with HCC, we found that positive IQGAP1 and negative IQGAP2 were associated with unfavorable disease-free and overall survival rates. This result suggested that IQGAP1 and IQGAP2 might be important prognostic markers for advanced stage HCC after curative hepatectomy. It is noteworthy that IQGAP1/2 switch acts as an independent prognositic factor in HCC. Further studies are needed to identify which protein plays the predominant role in HCC, thus enhancing their clinical predicative value for the prognosis of HCC patients.

In summary, this study demonstrated for the first time that positive IQGAP1 and negative IQGAP2 expression were correlated with advanced HCC stage and unfavorable

prognosis. We recommend that IQGAP1 and IQGAP2 should be used as adjunctive biomarkers to improve prognostication for individual patient. However, there is a fundamental problem in using immunohistochemicl staining in evaluating the expression profile of IQGAP1/2 as subjectively interpreted by histopathologists. Prospective studies with larger patient populations need further investigating the value of IQGAP1 and IQGAP2 by PCR and western blotting, and the cut-off value of IQGAP1/2 ratio as prognostic predictors.

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