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Original Article

Decreased expression of iron regulatory protein-1 in hepatocellular carcinoma associates with poor prognosis

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Abstract: Background: Iron regulatory protein-1 (IRP1) plays a critical role in cellular iron homeostasis and in the TCA cycle, which are closely associated with cancer development. However, the diagnostic and prognostic value of IRP1 in hepatocellular carcinoma (HCC) was not still estimated. Therefore, it was necessary to verify its expression and significance in patients with HCC in this study. Methods: The expression of IRP1 was analyzed in 42 paired HCC samples (HCC tissues *vs* matched adjacent non-cancerous liver tissues) and 191 paraffin-embedded HCC sections using immunohistochemistry (IHC). Then, receivers operating characteristic curve (ROC), Kaplan-Meier and Cox regression analysis were applied to evaluate the potential diagnostic and prognostic value of IRP1, respectively. Results: IRP1 expression level was significantly decreased in HCC tissues compared to the corresponding adjacent nontumorous liver tissues (P = 0.0003). Further correlation analyses indicated that the expression of IRP1 was significantly associated with TNM stage (P = 0.008) and vascular invasion (P = 0.008). ROC analysis showed the AUC was 0.765, and the optimal cutoff value of integrated optical density was 40860000, providing a sensitivity of 64.29% and a specificity of 76.19%. Moreover, overall survival (OS) and time to recurrence (TTR) analyses showed HCC patients with low IRP1 expression had lower survival rate (P = 0.007) and higher recurrence rate (P = 0.033). Furthermore, multivariate analysis showed that IRP1 was an independent prognostic factor for TTR (HR = 0.616, 95% CI = 0.398-0.952, P = 0.029). Conclusion: Collectively, our study demonstrated that IRP1 could be served as a potential diagnostic and prognostic marker for HCC patients.

Keywords: IRP1, hepatocellular carcinoma, prognosis, ROC

Introduction

Despite the great improvement on traditional treatments, such as surgery supplemented with radiotherapy and chemotherapy in recent years, hepatocellular carcinoma (HCC) is still the third major cause of cancer-related death globally [1]. Altered gene expression continuously involves in the development and progression of HCC, and regulates the malignant transformation, apoptosis, proliferation and metastatic capability of tumor cells [2]. It is critical to identify a dependable prognostic biomarker to accurately predict the outcome of HCC patients, especially among these abnormal expression genes. Although intensive efforts have been made to identify diagnostic and prognostic markers of HCC, and several biomarkers are being reported currently [3-5], valuable biomarkers are still urgently needed.

Iron regulatory proteins (IRPs) play a critical role in iron homeostasis [6]. In most mammalian cells, the plasma iron carrier transferring binds to the cell surface transferrin receptor 1 (TfR1), undergoes endocytosis, and releases iron in the acidified endosome [7]. Then, released iron is utilized in various functional metalloproteins or stored and detoxified in the cytosolic storage protein ferritin [8, 9]. IRPs, including IRP1 and

IRP2, bind to iron-responsive elements (IREs) and coordinately control the expression of TfR1 and ferritin by posttranscriptional mechanism [10]. In mammalian cells, IRP1 contains a 4Fe-4S cluster and IRP-2 does not [11, 12]. In addition to IRE-binding state, IRP1 could convert to a non-binding form with aconitase activity in iron-replete cells, which requires an intact 4Fe-4S cluster [13]. Therefore, IRP1, also known as aconitase 1 (ACO1), functions as a regulator of TCA cycle. Moreover, reactive oxygen species (ROS) affect cellular iron metabolism through regulating IRP1 activity [14, 15]. A growing body of research suggested that IRP1 was closely related to cancer development and progression. It was reported that overexpression of the constitutive $IRP1_{C437S}$ mutant, which was unable to form an iron-sulfur cluster, misregulated iron metabolism in human H1299 lung cancer cells [16]. Further research showed that the overexpression of IRP1 (either mutant or wildtype) did not alter the proliferation of the H1299 cells *in vitro*, but dramatically suppressed growth of tumor xenografts in nude mice [17]. In pancreatic cancer, SIRT3 inhibits tumor growth by regulating IRP1 activity and modulating cellular iron metabolism [18]. IRP1 is also known to inhibit the translation of HIF2α, which mediated biochemical processes of some anti-tumor compounds (such as Tempol and PGJ_2) [19, 20]. Another study suggested that control of intracellular iron by IRP1 might cause gamma ray-specific radioresistance in leukemia cells [21].

However, the prognostic significance of IRP1 in HCC is hitherto unknown. In this study, we verified the expression of IRP1, and evaluated its prognostic significance in HCC patients. Our data indicated that IRP1 was remarkably increased in HCC and could be served as a promising biomarker of HCC prognosis.

Materials and methods

Patients and specimens

A total of 181 cases of paraffin-embedded pathological HCC specimens which were randomly recruited at the Department of Hepatobiliary Surgery: the First Affiliated Hospital of Wenzhou Medical University; the Department of General Surgery at The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University from January 2006 and December 2009. This retrospective cohort consisted of 158 (87.3%) males and 23 (12.7%) females. Postsurgical survival data were available for all 181 patients. Another 42 paired HCC resection tissues and the corresponding adjacent liver tissues were also randomly recruited. Hepatitis B virus (HBV) infection was defined after HBV surface antigen (HBsAg) in the serum was detected using ELISA analysis. Computed tomography (CT) and magnetic resonance imaging (MRI) were used to verify tumor recurrence in suspected cases. In addition, an elevated serum AFP level (>20 ng/ml as positive) was further used to confirm tumor recurrence. No patients in this study received adjuvant therapies before surgery. Tumor stage was defined according to tumor-node metastasis (TNM) classification of the American Joint Committee on International Union against Cancer. The grade of tumor differentiation was assessed according to Edmonson and Steiner grading system. For the use of these clinical materials in this study, prior patients' consents and approval from the Institutional Research Ethics Committee of The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University were obtained.

Tissue microarray (TMA) construction

Two tissue microarrays were constructed according to the method described previously [22]. One TMA contained 181 HCC samples, and the other TMA contained 42 paired HCC and adjacent nontumorous tissues. In brief, all specimens were embedded in paraffin after 10% formalin fixing. The corresponding histological H&E-stained sections were reviewed by two pathologists to mark out representative areas. Then, each tissue core with a diameter of 0.6 mm was punched from the marked areas and re-embedded tissue samples were arrayed using a tissue-arraying instrument (Beecher Instruments, Sliver Spring, MD).

Immunohistochemical (IHC) analysis

IHC analysis for IRP1 was carried out similarly to previously methods [23]. Briefly, TMAs were incubated with anti-IRP1 antibody (ab 62701, 1:50, Abcam, Cambridge, MA, USA) overnight at 4°C. Photographs of three representative fields were randomly captured with 200X magnification. Using Image-Pro Plus v6.0 software, inte-

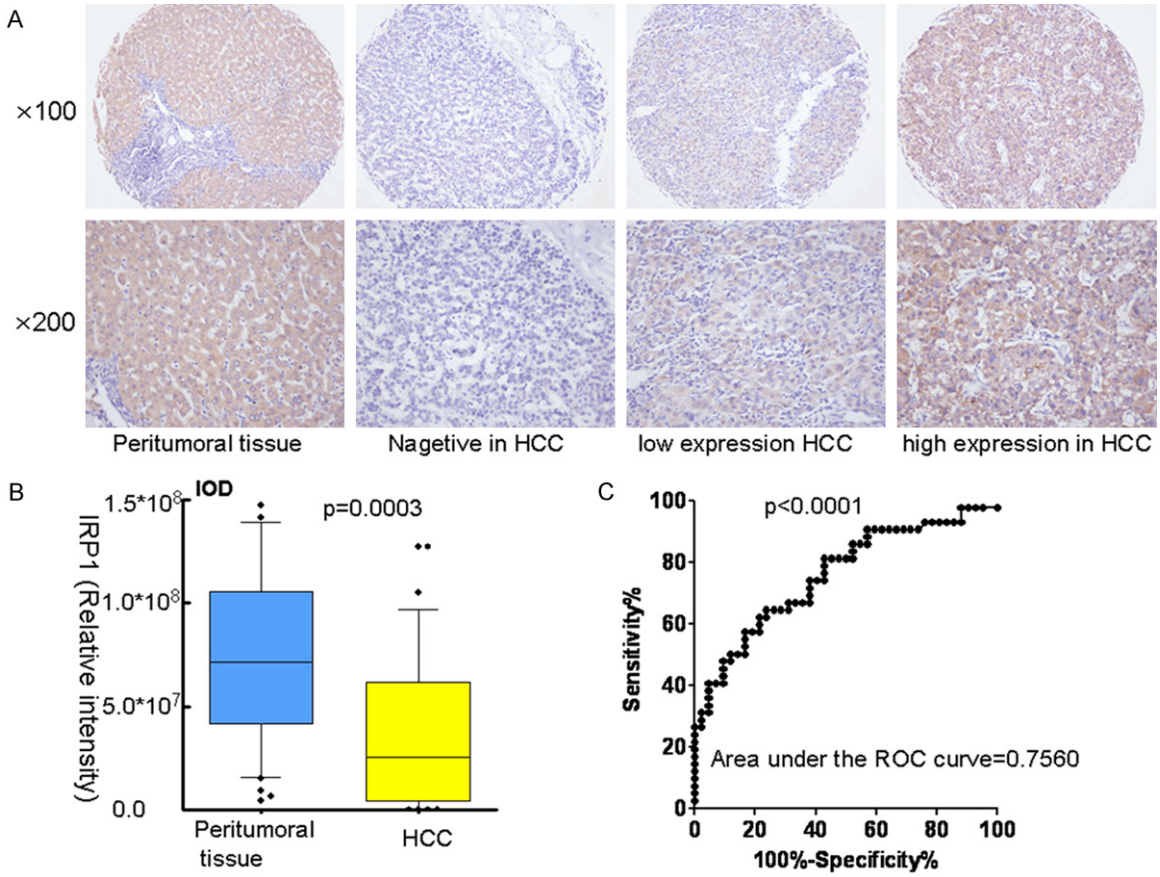

Figure 1. Decreased IRP1 expression in HCC tissues. IRP1 expression analyses in 42 paired samples of HCC tissues and matched peritumoral liver tissues using IHC staining. A. Representative images of the primary HCC tissue and matched peritumoral tissue sample taken from the same HCC case (negative staing) and low and high expression cases (magnification: × 100 and × 200). B. Integrated optical density (IOD) for IRP1 was obtained from 42 paired samples of HCC tissues and matched peritumoral liver tissues. C. Receiver-operating characteristic analysis showing the pixel density value of IRP1 in HCC. At a cut-off IOD level of 40860000, the pixel density value of IRP1 exhibited 64.29% sensitivity and 76.19% specificity for detecting HCC. Area under the ROC curve (AUC): 0.7650, 95% CI: 0.6526-0.8593, P < 0.001.

grated optical density (IOD) was counted and measured. The sum of intensity was used as final score of expression level.

Statistic analysis

Differences among two variables were assessed by two-tailed Student *t* test. Analyses of the receiver operating characteristic (ROC) were conducted to determine the cut-off value of the ratio of pixel density of IRP1 for diagnosing HCC. The chi-square test was used to analyze the relationship between IRP1 level and clinicopathologic features. OS and TTR curves were plotted by the Kaplan-Meier method and compared using the log-rank test. Multivariate analyses were performed by multivariate Cox proportional hazard regression model. Differences were considered to be statistically significant for $P < 0.05$.

Results

Decreased IRP1 expression in HCC tissues

IHC was performed to determine IRP1 expression in a tissue microarray (TMA), which enrolled 42 paired HCC tissue samples. In the **Figure 1A**, we showed that majorities of peritumoral tissue which showed strong and diffuse IRP1 cytoplasmic staining and negative, low expression, high expression of HCC cases (shwoed × 100 and × 200 magnification respectively). Compared with paired peritumoral tissue, HCC tissue showed significantly weaker IRP1 staining (**Figure 1B**). Quantitative IHC analysis re-

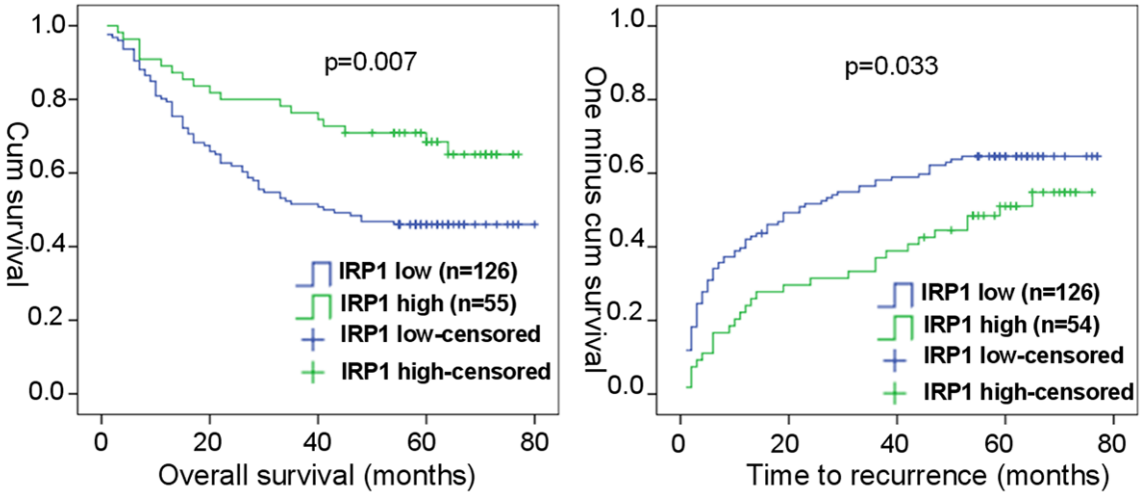

Figure 2. Correlation of low IRP1 level with unfavorable OS and TTR in HCC patients. Probabilities of OS A and TTR B of 181 total HCC patients were analyzed using Kaplan-Meier survival analysis (log-rank test).

vealed that the IOD values of IRP1 staining in all HCC tissues were lower than that in peritumoral tissues (P = 0.0003, paired t test, two-tailed). In addition, receiver-operating characteristic (ROC) analysis showed the AUC was 0.7560, and the optimal cutoff IOD value was 40860000, providing a sensitivity of 64.29% and a specificity of 76.19% (**Figure 1C**).

Prognostic significance of IRP1 expression in HCC patients

To determine the prognostic value of IRP1 in postsurgical HCC patients, Kaplan-Meier overall survival (OS) and time to recurrence (TTR) analyses were conducted. A retrospective cohort comprising 181 HCC cases was randomly collected to construct a TMA to determine IRP1 expression using IHC analysis. As shown in **Figure 2A**, the mean OS were 46.8 months for patients with low IRP1 expression and 59.6 months for patients with high IRP1 expression. As shown in **Figure 2B**, the mean TTR were 35.7 months for patients with low IRP1 expression and 48.1 months for patients with high IRP1 expression. These statistic results indicated that low IRP1 expression patients had much shorter OS times (P = 0.007) and a higher tendency of disease recurrence (P = 0.033), compared with high IRP1 expression patients.

Correlation of IRP1 expression with clinicopathological features

To further investigate the relationship between physiological or pathological variables and expression of IRP1, the clinicopathological data of all HCC patients detected in our study (n = 223) are summarized in **Table 1**. The results showed that significant correlations (chi-square test) were found between IRP1 expression and two parameters including TNM stage (P = 0.008) and vascular invasion (P = 0.008). HCC patients with low IRP1 expression had a higher tendency to be with advanced TNM stage and frequent vascular invasion. However, No significant associations were observed between IRP1 expression and other clinicopathological parameters such as such as age, sex, HBsAg, liver cirrhosis, serum APF, tumor differentiation, tumor number, tumor size and Child-Pugh class (all P > 0.05, **Table 1**).

Univariate and multivariate analyses of prognostic power of IRP1 in HCC patients

To identify prognostic significance of IRP1 and other clinicopathologic parameters in HCC patients, Cox regression analysis was performed in the retrospective cohort. Univariate Cox analysis indicated that serum APF, TNM stage, tumor size, tumor number, vascular invasion, and IRP1 level were potential candidates for OS and TTR (all P < 0.05). Next, those clinicopathologic variables significant in Cox univariate analysis were further evaluated in Cox multivariate proportional hazards regression analysis. As shown in **Table 2**, IRP1 was an independent predictor for TTR (HR: 0.616, 95% CI: 0.398-0.952, P = 0.029).

Table 1. Correlation between IRP1 expression and clinicopathologic parameters in HCC

Variable		IRP1 Low	IRP1 High	p
Sex				0.804
	Male	125	69	
	Female	18	11	
Age				0.802
	≤50	69	40	
	>50	74	40	
HBsAg				0.161
	Negative	24	20	
	Positive	116	60	
Serum AFP				0.212
	≤20 ng/ml	43	31	
	>20 ng/ml	98	49	
Liver cirrhosis				0.126
	No	42	16	
	Yes	101	64	
TNM				0.008
	I	34	35	
	II	81	34	
	III-IV	28	11	
Child-pugh class				0.566
	A	129	74	
	B	14	6	
Tumor size				0.090
	≤5 cm	60	43	
	>5 cm	83	37	
Tumor number				0.439
	Single	106	63	
	Multiple	37	17	
Tumor differentiation				0.669
	Well	11	9	
	Moderate	128	69	
	Poor	4	2	
Vascular invasion				0.008
	No	41	37	
	Yes	102	43	

Note: Chi-square test for comparison between groups. HBsAg, hepatitis B surface antigen; AFP, alpha-fetoprotein; TNM, tumor-node-metastasis.

Discussion

In the present study, to elucidate the clinical role of IRP1 in HCC, we applied TMA and IHC to examine its expression in a retrospective cohort of HCC patients. Our results demonstrated that IRP1 expression was significantly decreased in HCC tissues and acted as a potent independent prognostic factor for HCC patients. To our knowledge, this is the first study to analyze IRP1 expression in HCC using TMA-based IHC method. However, there is no study so far that discusses the molecular mechanism of IRP1 in HCC progression.

Cellular iron homeostasis is essential for cellular processes and many cancer cells exhibit dysregulation in iron metabolism. Maintenance of cellular iron homeostasis is regulated by IRPs, which include IRP1 and IRP2. IRP1 is a bifunctional protein that functions as an essential enzyme in the TCA cycle and IRE binding protein to control the iron levels. Its expression and activity is regulated by cellular iron levels, cellular reactive oxygen species (ROS) and hypoxia [14, 24, 25]. Several researchers have suggested that IRP1 involved in cancer progression and anti-cancer drug resistant. Overexpression of IRP1 (either mutant or wildtype) dramatically suppressed growth of lung cancer xenografts in nude mice *in vivo* [17]. In pancreatic cancer, SIRT3 inhibits tumor growth by regulating IRP1 activity and modulating cellular iron metabolism [18]. Moreover, upregulation of IRP1 binding activity to IRE by PGJ2, an endogenous cellular metabolite, could inhibit HIF2a translation within tumor epithelial cells or mesenchymal cells of the tumor microenvironment [20]. It indicated IRP1 also might show anti-inflammatory and putative anti-neoplastic effects. In results of Kaplan-Meier survival analysis, our data showed that patients with high IRP1 expression had longer survival. This might be explained in part by rapid growth rate and dyregulation in iron metabolism, due to IRP1 downregulation in HCC. Meanwhile, Cox regression analysis suggested IRP1 as an independent prognostic factor, indicating that IRP1 may be a pivotal modulator involved in HCC development.

Metabolic reprogramming, such as the alterations of activity and expression in tricarboxylic acid (TCA) cycle key enzymes, is primary hallmarks of many malignant tumors including HCC [26]. Most cancer cells predominantly produce energy via glycolysis followed by lactic acid fermentation [27], rather than by glycolysis followed by oxidation of pyruvate in mitochondria as in most normal cells even if oxygen is plentiful [28]. Inactivation of the TCA cycle enzyme

Table 2. Univariate and multivariate analysis of different prognostic parameters in patients with HCC by Cox regression analysis (n = 181)

	OS				TTR			
		Multivariate				Multivariate		
Factors	Univariate *p*	HR	95% CI	*p*	Univariate *p*	HR	95% CI	*p*
Sex: Male *vs* Female	0.592				0.685			
Age: ≤50 *vs* >50	0.339				0.869			
HBsAg: positive *vs* negative	0.167				0.057			
Serum AFP (ng/ml): ≤20 *vs* >20	0.004	1.830	1.105-3.033	0.019	0.016			
Liver cirrhosis: yes *vs* no	0.066				0.010	1.788	1.122-2.850	0.015
TNM: I *vs* II *vs* III-IV	0.000	2.110	1.493-2.980	0.000	0.000			
Child-pugh: A *vs* B	0.313				0.615			
Tumor size: ≤5 *vs* >5	0.000				0.000	1.908	1.284-2.834	0.001
Tumor number: single *vs* multiple	0.000				0.000	2.622	1.723-3.988	0.000
Tumor differentiation: well *vs* moderate *vs* poor	0.180				0.184			
Cascular invasion: no *vs* yes	0.003				0.009			
IRP1: low *vs* High	0.008				0.038	0.616	0.398-0.952	0.029

Note: Chi-square test for comparison between groups. HBsAg, hepatitis B surface antigen; AFP, alpha-fetoprotein; TNM, tumor-node-metastasis.

could drives a metabolic shift to aerobic glycolysis in cancer cells. For example, Inactivation of the TCA cycle enzyme, fumarate hydratase (FH) activates the anabolic factors, acetyl-CoA carboxylase and ribosomal protein S6 in FH-deficient kidney tumors and cell lines from patients with hereditary leiomyomatosis renal cell cancer (HLRCC) [29]. Similarly, reduced expression or loss of SDHD, which catalyzes the oxidation of succinate to fumarate, is observed in human cancers including gastric and colon carcinoma [30, 31]. Due to SDHD down-regulation, succinate is accumulated and therefore increases expression of genes that facilitate metastasis and glycolysis, ultimately leading to tumor progression [32]. When cellular iron levels are high, IRP1 could assemble a 4Fe-4S cluster that prevents IRE-binding and and converts it to cytosolic aconitase, which catalyzes the stereo-specific isomerization of citrate to isocitrate via the intermediate cis-aconitate during the citric acid cycle [13]. In addition, the (4Fe-4S) cluster of IRP1 is also stabilized by hypoxia [33, 34]. It has been reported that the abnormal expression and activity of IRP1 contributed to tumorigenesis of the prostate and malignant characteristics of human prostate carcinoma [35, 36]. In our study, we found that IRP1 expression was significantly decreased in clinical HCC tissue. We speculated that this change might further weaken and impair aerobic function of mitochondrial, while enhance the glycolytic rates in HCC, leading to form a rapidly growing tumor cells. However, the enzyme activity of RIP1 has not been proved in this study. And it is still unclear that IRP1 down-regualtion whether effects the glycolysis process in HCC.

Taken together, our study provides compelling clinical evidence that IRP1 could be served as a potential diagnostic and prognostic marker for HCC patients. The limitations of this study included small sample size, lack of function investigation, and mechanism unclear. Therefore, further studies were needed to explore the role of IRP1 in HCC progression. In this study, our results indicated IRP1 expression evaluated by IHC could be used as an additional tool in identifying those patients at risk of HCC progression.

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Disclosure of conflict of interest

None.

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