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Original Article

Correlation of LARP1 and E-cadherin expression with prognosis of intrahepatic cholangiocarcinoma

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Abstract: Objective: The abnormal expression of LARP1 and E-cadherin (E-cad) is related to tumor occurrence and metastasis. Our study analyzed the expression of LARP1 and E-cad in intrahepatic cholangiocarcinoma (ICC) and investigated the prognostic value of the two proteins. Methods: Immunohistochemistry was performed to detect the expression of LARP1 and E-cad in 50 ICC clinical specimens with adjacent normal tissues and 20 normal bile duct tissues. In situ hybridization was performed to analyze the expression of LARP1 mRNA in all the specimens. Results: LARP1 protein and mRNA expression levels in ICC tumor tissues were significantly higher compared with corresponding adjacent normal tissues and normal epithelial tissues ($P<0.01$). E-cad protein expression in ICC tumor tissues was remarkably lower than that of the adjacent normal tissues and benign bile duct tissues ($P<0.01$). Correlation analysis demonstrated that LARP1 and E-cad expression levels were significantly related with the tumor-node-metastasis staging and lymph node metastasis ($P<0.01$), while no correlation was observed with patient age, gender, and tumor size. Moreover, Spearman rank correlation test revealed that LARP1 expression was negatively related to E-cad ($P<0.05$). More importantly, the patients with higher LARP1 expression or lower E-cad expression had a shorter overall survival postoperatively than those with LARP1 lower expression or E-cad higher expression. Multivariate analysis demonstrated that LARP1 and E-cad were both considered as important prognostic factors for survival time. Conclusion: These findings suggest that the abnormal expression of LARP1 and E-cad showed a close relationship with the occurrence and metastasis of ICC, leading to poor prognosis indirectly.

Keywords: LARP1, E-cadherin, intrahepatic cholangiocarcinoma

Introduction

Intrahepatic cholangiocarcinoma (ICC) that occurs in the intrahepatic bile duct (the first grade above the left and the right hepatic duct) is considered as one of the primary hepatobiliary cancers with high-grade malignancy and poor prognosis. In recent years, ICC accounted for 10%-20% of the newly diagnosed liver cancers, and has become the second largest, only after HCC, of all the malignant liver tumors [1]. With the development of medicine, several new therapeutic strategies have appeared [2]. However, surgery remains the preferred method for the treatment of cholangiocarcinoma clinically [3]. As there are no specific clinical symptoms or tumor markers, many patients miss the best time for surgery once they are diagnosed with cholangiocarcinoma. Therefore, it is necessary to find novel effective biomarkers for improving the early diagnosis of ICC [4].

The La-related protein 1 (LARP1) is an RNA binding protein that plays a significant role in many physiological processes like embryogenesis and cell cycle [5]. Recently, related molecular studies have indicated that LARP1 protein mediates protein translation as a molecular switch [6]. It has increased expression in some malignant tumors [6, 7], but whether it is associated with abnormal expression in ICC and its correlation with the prognosis of ICC patients were still ambiguous.

E-cadherin (E-cad) is a transmembranous glycoprotein widely expressed in epithelial cells. It plays an important role in keeping the shape and stability between epithelial cells [8]. Several studies have proved that abnormal expression of E-cad protein was associated with epithelial-mesenchymal transition (EMT) process in many cancers, including lung cancer and breast cancer [9, 10]. In our study, we mainly

Table 1. Human mRNA gene sequence of LARP1

Name	mRNA gene sequence
LARP1	(1) 5'-ACATGAACAACATCACCTACTACTTTGACAATGTC-3'
	(2) 5'-AGGTCATTAATGATGGCCTCTTCTACTATGAGCAG-3'
	(3) 5'-TCACACAACACGTCTACCATAAGTATCGTAGGCGC-3'

detected the expression of LARP1 and E-cad in ICC, studied their association, and analyzed their relationship with the clinicopathological features to investigate whether for any potential value in predicting the prognosis of cholangiocarcinoma.

Materials and methods

Patients and clinicopathological data

From January 2005 to September 2014, we collected data of 50 patients with confirmed ICC and 20 patients with hepatic hemangioma. All patients underwent a complete curative resection at the First Affiliated Hospital of Anhui Medical University, including 21 males and 29 females, with age ranging from 28 to 72 years (mean 56 years). Study patients received no chemoradiation before surgery, and the diagnoses were confirmed by at least two pathologists. The patients' medical records included information regarding gender, age, capsule integrity, tumor diameter, venous tumor thrombus, lymph node metastasis, and TNM staging. Among them, tissue 2 cm was sampled as tumor adjacent tissue, and hepatic hemangioma patients served as a control. As of July 2017, 40 patients were followed-up through postoperative review or telephone inquiry for at least 12 months or until death. The period of postoperative survival varied from 2 months to 63 months (median 26 months). Among the other 10 patients, 5 patients died due to postoperative bleeding or infection. The remaining patients who died due to other causes or lost to follow-up were defined as censored. The study was approved by the Ethics committee of Anhui Medical University and informed consent from all the patients was obtained.

Immunohistochemistry

Immunohistochemistry was performed according to the manual of a two-step protocol. Slices were placed in a baking machine for 20 minutes, and then were successively placed in xylene and gradient alcohol (100%, 70%, and 50%), for the purpose of dewaxing and rehydration. 3% H_2O_2 was used for blocking the activity of endogenous peroxidase. After the following steps, the sections were washed with 0.01 mol/L of phosphate buffered saline (PBS) three times for 3 minutes. The slices were then immersed in 0.01 mol/L sodium citrate buffer and placed in a microwave oven for 20 minutes for antigen retrieval and cooled to room temperature. The sections were then covered with anti-LARP1 polyclonal antibody (1:250 dilution, Abcam Biotechnology, USA) overnight at 4°C. Next, a secondary antibody marked with horseradish peroxidase (mouse anti-rabbit-IgG; Zhongshan Golden Bridge Biotechnology Co., Ltd.) was used for 10 minutes at 37°C. After washing, the sections were stained with a diaminobenzidine liquid system, counterstained with Mayer's hematoxylin, and then mounted. Anti-E-cadherin polyclonal antibody (MXB, Fujian; working solution) was stained as described previously. For each case, PBS was used instead of an antibody to set the negative controls, and without anti-LARP1 or anti E-cadherin polyclonal antibody to set the blank control.

In situ hybridization

The experimental oligonucleotide probe of LARP1mRNA (showed in **Table 1**) was designed by Boster Biotechnology (Wuhan, China), and procedures were carried out in accordance with the kit instructions. Firstly, after gradient xylene and ethanol treatment, pepsin (diluted by 3% citric acid) was poured on the slices under room temperature for 20 minutes to expose its mRNA nucleic acid fragments. Then, 1% formaldehyde was used for fixation. This is followed by rinsing in distilled water for 15 minutes. Prehybridization solution and hybridization solution were added on the sections overnight at 42°C, and PBS was used instead of hybridization solution to set the negative control. After that, sealing fluid, biotinylated digoxin, SABC-POD, biotin peroxidase were dripped successively at 37°C after washing it with PBS for three times. Finally, all the sections were stained by DAB and hematoxylin staining. Then the sections were dehydrated and mounted using standard procedure.

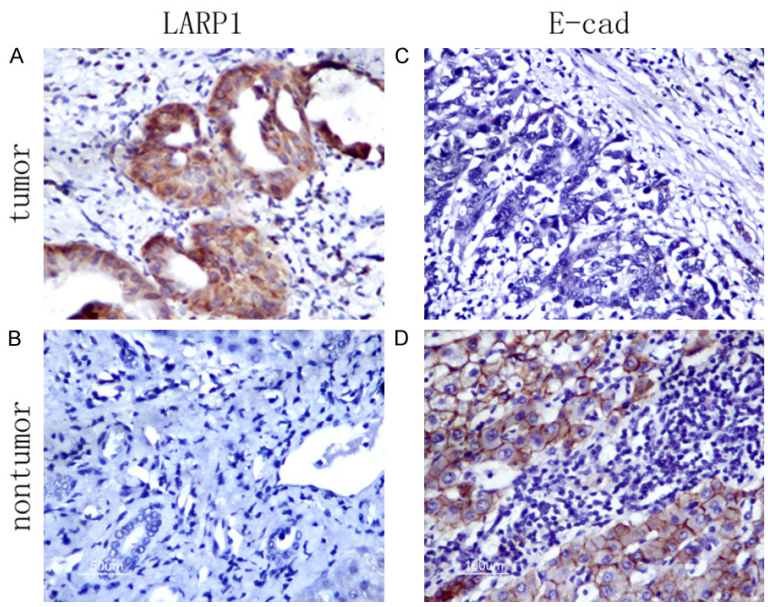

Figure 1. Immunohistochemical staining of LARP1 and E-cad protein in ICC specimen tissues, adjacent normal tissue, or normal epithelial tissues. A. LARP1 protein staining was strong in ICC tissue; B. Negative staining of LARP1 in adjacent tissue and in normal tissue samples; C. Negative staining of E-cad protein in ICC tissues; D. E-cad staining was strong in adjacent tissue and in normal tissue samples (PV × 400).

Evaluation of immunohistochemical staining and in situ hybridization

The assessment of immunohistochemistry staining evaluated positive expression of LARP1 in the cytoplasm, while E-cad was positively expressed on the cell membrane. Immunohistochemical staining score was recorded by 3 different experienced pathologists. Final results were determined by analyzing the staining intensity and percentage of positive cells. The staining intensity of the section was given a score of: no color (0 point), light yellow (1 point), pale brown (2 points), dark brown (3 points). The percentage of positive cells was given a score of: 0 point (0-10%), 1 point (10%-40%), 2 points (40%-70%) and 3 points (>70%). By multiplying the above two items, ≤3 points was considered as low expression, while >3 points was considered as high expression.

The score for in situ hybridization was obtained by three different experienced pathologists. Under the microscope, a positive results for LARP1stained brownish yellow. Three different visual fields were selected to observe the positive expression intensity and positive rate (× 200). Semiquantitative analysis was used to evaluate the percentage of positive cells and staining intensity. First, staining intensity was given a score of: 0 points for no significant staining, 1 point for slight yellow, 2 points for moderate yellow and 3 points for strong yellow color. The percentage of positive cells was given a score of: 0 to 5% as 0 points, 6% to 25% as 1 points, 26% to 50% as 2 points, 51% to 75% points as 3 points, and >75% as 4 points. The final results were determined by adding the two scores together: 0 is considered as negative (-), 1~3 as weakly positive, 4~5 as moderately positive, and 6~7 as strongly positive.

Statistical analysis

All the data analysis was completed by using SPSS19.0 software. Chi square test was applied to estimate the relationship between the expression of LARP1 and E-cad and the clinicopathological data of each group. Analysis of the correlation between LARP1 and E-cad was obtained by using Spearman method. Kaplan-Meier survival analysis and log-rank test was used to determine the overall survival rate of patients with different protein expression levels after surgery. To further explore independent indicators that affect the patient's life cycle, we established a Cox proportional hazards regression model with multivariable analysis. P values <0.05 are considered statistically significant.

Results

Expression of LARP1 and E-cadherin in cholangiocarcinoma

Immunohistochemical staining of all sections revealed that LARP1 protein was mainly present in the cytoplasm of ICC patients, while E-cad was mainly present on the cell membrane (**Figure 1**). The expression of LARP1 in cancer

Table 2. Clinicopathological correlation of LARP1 and E-cad expression in 50 patients with ICC

Parameters	No.	LARP1 expression		X^2	*P*	E-cad expression		X^2	*P*
		+	-			+	-		
Age (years)									
≤50	20	11	9	2.589	0.108	9	11	0.347	0.556
>50	30	23	7			11	19		
Gender									
Male	21	15	6	0.196	0.658	10	11	0.876	0.349
Female	29	19	10			10	19		
Capsule integrity									
Yes	18	13	5	0.230	0.631	8	10	0.231	0.630
No	32	21	11			12	20		
Tumor size									
≤5 cm	22	14	8	0.344	0.558	11	11	1.637	0.201
>5 cm	28	20	8			9	19		
Venous cancer embolism									
Yes	16	11	5	0.006	0.938	4	12	2.206	0.137
No	34	23	11			16	18		
Lymph node metastasis									
Yes	24	21	3	8.065	0.005	5	19	7.065	0.008
No	26	13	13			15	11		
TNM stage									
I~II	20	8	12	12.010	0.001	13	7	8.681	0.003
III~IV	30	26	4			7	23		

tissues was significantly higher than that of the adjacent and normal tissues ($P<0.01$). In contrast, E-cad in adjacent and normal tissues showed a higher expression compared with cancer ($P<0.01$). There was no statistical significance in the expression of the two proteins between the paracancerous tissues and the normal tissues ($P>0.01$).

Correlation of LARP1 and E-cadherin expressions with clinicopathological information

In order to detect the relationship between LARP1 and E-cad in different tissues, clinicopathological data of the patients were obtained. A chi square test was performed and the results showed that the expression levels of LARP1 and E-cad were related to lymph node metastasis and clinical TNM staging of the patient ($P<0.01$), while no significant correlation was observed with patient age, gender, membrane integrity, tumor diameter ($P>0.05$) as shown in **Table 2**. Furthermore, Spearman's rank-order correlation coefficient showed that the expression of LARP1 was negatively correlated with E-cad ($P<0.05$).

Expression of LARP1mRNA in intrahepatic bile duct tissues

As illustrated in **Figure 2**, different levels of LARP1 mRNA expression were detected in tumor tissues, adjacent tissues, and normal tissues. Expression level of LARP1 mRNA in tumor specimen tissues was significantly higher than that in adjacent normal tissues and normal epithelial tissues ($P<0.05$). The outcomes were consistent with the results obtained by immunohistochemistry.

Survival analysis

Kaplan-Meier survival curve was used to evaluate the effect between the various expression of LARP1 in different intrahepatic bile duct tissues with patient life cycle at different phases. A total of 40 ICC patients was divided into a high expression group and a low expression group according to the LARP1 expression in different tissues. The survival curve demonstrated that the group with higher expression of LARP1 showed a weaker prognosis than that of the lower expression group (**Figure 3**). The bias

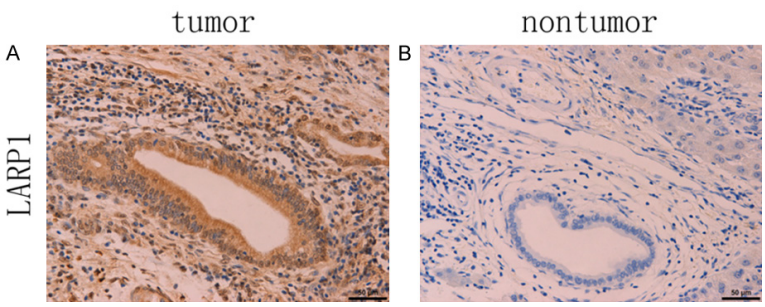

Figure 2. In situ hybridization staining of LARP1mRNA in ICC tissues, adjacent tissues, or normal tissues. A. LARP1mRNA staining was strong in ICC tissues; B. Negative staining of LARP1mRNA in adjacent tissue or in normal tissue samples. ISH data and IHC data used consecutive tissue sections with the same morphology (PV × 400).

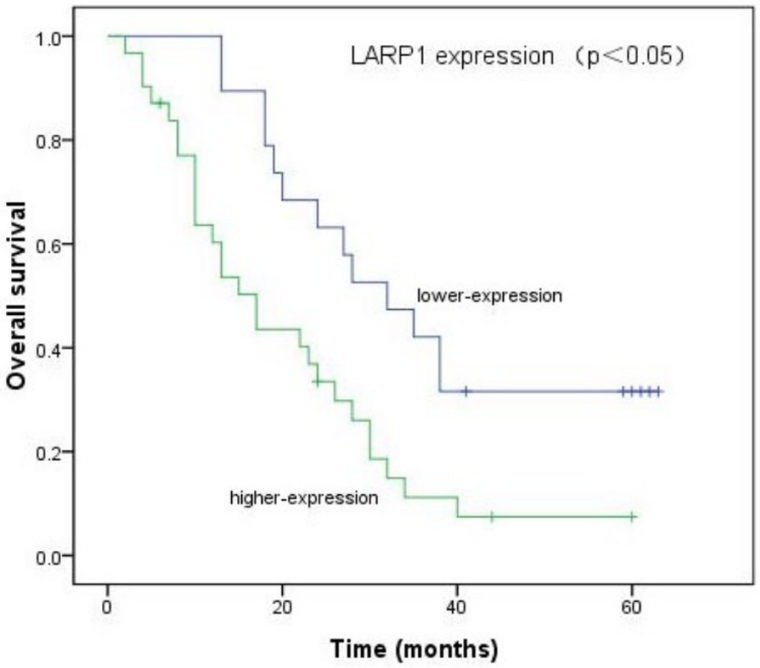

Figure 3. Kaplan-Meier survival analysis of OS in ICC patients, according to LARP1 expression level.

was verified by Log-Rank test, which showed that LARP1 expression was significantly related to the survival time in patients diagnosed with ICC (Log-Rank, *P*=0.013/0.05).

Univariate analysis and multivariate Cox regression analysis

For further detection of the markers that affected the prognosis of patients with ICC, a univariate and multivariate Cox regression analysis with patient clinical data were performed. Univariate analysis revealed that significant indicators of strong correlation with prognosis are as follows: LARP1 overexpression, E-cad low-expression, TNM staging and lymph node metastasis. However, multivariate analysis showed that only LARP1 expression, E-cad expression, and TNM staging were independent factors affecting the prognosis of ICC. Among them, LARP1 served as a risk element while E-cad served as a protective element (**Table 3**).

Discussion

Due to the distinct anatomy and pathology of ICC, the incidence of ICC has increased rapidly in recent years [11]. Cholangiocarcinoma has a greater degree of malignancy because of its obscure symptoms, early metastasis, high recurrence rate, and poor prognosis after surgical resection [12, 13]. Currently, with the progression of advanced radiological imaging technology and the emergence of new anti-tumor drugs, prevention and treatment of ICC patients have been improved to some extent [2]; however, the survival rate remains lower than other malignancies [14]. Therefore, more effective new molecular biomarkers are vital for the early diagnosis and treatment of patients with ICC.

LARP1 is an important RNA binding protein that combines with mRNA via the LA-motif, and plays an important role in regulating the stability of mRNA [15]. It is widely found in animals, plants, and various microorganisms. LARP1 is a member of the five LARP protein families present in humans. Relevant studies have shown that LARP1 was involved in several physiologic processes, such as cell division, migration and apoptosis [16, 17]. It acts as a cofactor by binding with other substances intracellularly during RNA degradation [18]. Additionally, the translation process of TOR-mRNAS is enhanced by

Table 3. Overall survival by Cox proportional hazards model

Variable	B	SE	Wald	Relative Risk (95% CI)	*P* (value)
Univariate					
Age (years)				0.939 (0.498-1.771)	0.847
Gender				1.183 (0.627-2.230)	0.604
Capsule integrity				1.403 (0.723-2.722)	0.317
Tumor size				1.092 (0.583-2.047)	0.783
TNM stage				4.365 (2.133-8.933)	<0.001
Lymph node metastasis				2.379 (1.247-4.535)	0.008
LARP1				2.492 (1.271-4.889)	0.008
E-cad				0.269 (0.128-0.567)	<0.001
Multivariate					
TNM stage	1.574	0.483	10.630	4.826 (1.874-12.432)	0.001
LARP1	0.913	0.386	5.601	2.491 (1.170-5.306)	0.018
E-cad	-0.972	0.415	5.470	0.378 (0.168-0.854)	0.019

intracellular phosphorylation [6, 19, 20]. It has been reported that the expression of LARP1 was increased in a variety of malignant tumors, and was associated with clinical prognosis. For example, Chan Xie examined the expression of LARP1 in 15 HCC cell lines and 272 clinical specimens using real-time PCR, immunohistochemistry, and western blot analysis [5]. Results showed that the levels of LARP1 were higher in HCC cell lines and survival analysis showed poor prognosis. However, there were no relevant reports of LARP1 expression in ICC. We used immunohistochemistry and In situ hybridization to demonstrate the role of LARP1, which showed an increased expression in ICC, and the expression levels were positively correlated with tumor TNM staging and lymph node metastasis. These results are consistent with other malignant tumors, such as cervical cancer.

Cadherin is a binding affinity and Ca^{2+} dependent cell adhesion glycoprotein. It plays an important role in cell recognition, migration, and tissue differentiation [21]. In addition, the development and metastasis of cancer involves the loss of function or expression of E-cadherin. A strong link between E-cadherin and the EMT process was observed in different tumor tissues. Studies have shown that the expression of E-cadherin was decreased during the process of EMT formation [9]. Downregulation of E-cadherin reduced the intensity of cell adhesion in an organism, leading to increased cell activity. Our results demonstrated that the expression of E-cadherin in ICC was significantly lower than that of adjacent tissues and normal intrahepatic bile duct tissues. The expression of E-cadherin was closely related to the metastasis and invasion of ICC, and had a close relationship with the tumor staging.

Through immunohistochemistry and in situ hybridization, LARP1 was mainly distributed in the cytoplasm, and the expression of cancer tissues was significantly higher than that in paracancerous tissue and normal tissue. However, E-cad was mainly distributed in the cell membrane and its expression in cancer tissues was lower than that in paracancerous and normal tissues. After comparing with the clinicopathologic data, the expression levels of LARP1 and E-cad were closely related with tumor TNM staging and lymph node metastasis, while no significant relationship was observed with patient age, gender, or tumor size. This provides new clues about the invasion and metastasis of ICC. Spearman method showed a negative correlation between LARP1 expression and E-cad expression. These findings are also consistent with the role of LARP1 in cell migration.

In order to comprehend the relationship between LARP1 expression and postoperative survival period, we used Kaplan-Meier survival analysis, which showed that patients with higher expression of LARP1 had a lower survival time and vice versa with low expression ($P<0.05$). Further analysis with univariate and multivariate Cox regression showed that TNM stage, LARP1 expression, and E-cad expression are independent factors affecting the survival time for patients undergoing surgical treatment. LARP1 is considered as a risk factor, while E-cad was a protective factor. Their expression provides a new means for the diagnosis of ICC and prognostic analysis, improving the accuracy of early diagnosis.

In conclusion, LARP1 as a potential biomarker provides new insights into the treatment of ICC,

but the mechanism of interaction with E-cad still remains unclear. Hence, the mechanisms for the intracellular regulation of LARP1 expression and its interaction with E-cad require further exploration.

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Disclosure of conflict of interest

None.

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