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Original Article

LCN1 is highly expressed in cholangiocarcinoma patients and indicates poor prognosis

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Abstract: Early detection of malignant biliary tract diseases, especially cholangiocarcinoma (CC) in patients, is very difficult and often comes too late to benefit the patient. Therefore, the goal of this study was to identify novel biomarkers to distinguish CC from nonmalignant lesions. Protein expression in the bile between patients with CC and cholelithiasis was compared by LC-MS/MS analysis. Lipocalin 1 (LCN1) was found to be significantly higher in the bile from CC patients than cholelithiasis patients. Enzyme-linked immunosorbent (ELISA) was performed to detect the status of bile LCN1 expression in patients with CC and with cholelithiasis. The level of LCN1 expression in the bile was significantly up-regulated in CC compared to cholelithiasis ($P<0.001$). LCN1 expression was also detected by Western blotting and immunohistochemistry in cells and tissues respectively. Expression of LCN1 was higher in CC cells than in normal choanocytes, and expression was also higher in CC tissues than the paired adjacent normal tissues. High LCN1 expression in CC was significantly correlated with tumor size, metastasis, and clinical stage. Additionally, LCN1 high-expressed CC patients had significantly shorter overall survival (OS) than those with low expression ($P=0.0309$). These findings indicate that LCN1 detection may have a benefit for CC diagnosis, and is associated with poor prognosis in CC.

Keywords: Cholangiocarcinoma, cholelithiasis, LCN1, proteomics

Introduction

Cholangiocarcinoma (CC) is a malignancy originating from bile duct epithelial cells and is associated with low five-year survival rate [1]. Surgical resection is the only effective treatment for CC, and the 5-year survival rate is only 5% after surgery, while <1% without surgical treatment [2]. Limited by image detection techniques, most patients are found at late stage and miss operation opportunity. Therefore, exploring novel biomarkers for CC diagnosis at early stage are important for the prognosis of CC patients.

The lipocalins are a family of small, soluble, secreted proteins, characterized by their typical fold of an antiparallel eight-stranded b-barrel, with an a-helix attached [3]. They bind and transport mainly hydrophobic molecules and take participant in a wide range of biological processes, such as immunity, signal transduction, and vision. Lipocalin 1 (LCN1) is a member of the lipocalin superfamily, binding to a variety of lipophilic ligands. Although originally isolated as a protein highly produced by the lacrimal and lingual salivary glands, it has since been found to be expressed by several other secretory tissues such as prostate, mucosal glands of the tracheobronchial tree, nasal mucosa, and sweat glands [4-9]. It has been demonstrated that LCN1 is involved in innate immune responses, which can act against inflammation. It has also been reported that LCN1 could prevent the oxidation reaction of lipid peroxidation products *in vitro* [10]. Moreover, biological relevance of its multiple roles has been reported, its main function seems to be eliminating of lipophilic, potentially harmful molecules, thus acting as a protection factor for cells and tissues [11].

Currently, the precise biological function of LCN1 has not been fully characterized and the relevance with the tumorigenesis is unknown.

In this study, protein expression in the bile between patients with CC and cholelithiasis was compared, and LCN1 was found to be significantly differentially expressed. LCN1 expression in CC tissues was higher than adjacent normal tissues, and expression of LCN1 correlated with tumor size, distant metastasis, and overall survival (OS). These findings provide novel role of LCN1 in CC tumorigenesis and progression.

Materials and methods

Patients

Bile samples and patient data collection were carried out in Second Hospital of Dalian Medical University, and all these patients with bile samples collected from patients with obstructive jaundice, bile duct cancer, or cholelithiasis in a total of 45 (20 with CC, 25 with cholelithiasis). Patients in both gallstone disease and bile duct cancer groups had no acute bile duct inflammation. Patients with bile duct carcinoma were confirmed by pathology. The study protocols were approved by the Hospital Ethics Committee of the Second Hospital of Dalian Medical University. Written informed consent based on the Declaration of Helsinki was obtained from the patients.

Liquid chromatography tandem mass spectrometry (LC-MS/MS)

All LC-MS/MS experiments were performed in the RPLC-MS/MS system. The system has four high performance liquid chromatography gradient pump, autosampler and linear ion trap Orbitrap mass spectrometry (LTQ-Orbitrap static Velos), using 4 cm long C18 Trap column (diameter 200 μm) auxiliary automatic sample, after the sample the sample was first into the reserved trap column, and then after gradient elution to a length of about 15 cm analytical column (diameter 75 μm), mass spectrometry detector. The sample was placed into the flow velocity gradient separation phase control at 75 μl/min. Reverse settings for 5 minutes at 0-5% mobile phase B (0.1% formic acid/acetonitrile) 90 minutes 5%-35%, the mobile phase B, 15 minutes 35%-80% B 80% in the mobile phase, the mobile phase B 10 minutes after irrigation, the whole system began to use 100% mobile phase A (0.1% formic acid) wash 20 minutes, to balance the system, the next generation of the sample.

LTQ mass spectrometry example transfer capillary temperature was set to 250 degree, the electrospray voltage setting for 2.2 KV, normalized collision energy settings for 35%, the activation time was 10 ms, and the mass resolution was set for scanning at 60000. All MS/MS spectrum acquisition used data dependent mode of collision induced dissociation to complete. MS scan setting for the full scan mass to charge ratios (M/Z range was dynamic 400-2500. Exclusion was set to repeat 2 times, with a duration for 30 seconds, and a dynamic exclusion time of 90 seconds. Xcalibur software (version number: 2.1, thermo) was used for system control and data acquisition.

All the LC-MS/MS data using mascot (Version 2.5, Matrix Science) for data retrieval, the bile protein sample selection the UniProt database retrieval. In the database search of cysteine, a retrieval set was a fixed modification and methionine was set as variable modification, peptides settings for trypsin total resection, the maximum allowed two missed cleavage sites appeared. The parent ion mass error setting 20 ppm and fragment ion mass error settings for false positive rate of 0.5 Da. Peptides were identified. Control in less than 1%.

Enzyme-linked immunosorbent (ELISA) assay

LCN1 levels of bile samples were measured by using the LCN1 ELISA kit (BPE11114, Lengton, Shanghai, China) according to the manufacturer's instruction. Briefly, samples were added to a 96-well plate coated with biotin labeled anti-LCN1 antibody and incubated for 1 hour at 37°C, and then incubated with HRP-streptavidin conjugate antibody for 1 hour at room temperature. Substrate solution was added and incubated for 20 minutes at room temperature in the dark, stop solution was added and the plates were read at 450 nm with a microplate reader. Serum tumor markers CEA and CA19-9 were also measured by the ELISA method.

Cell culture

Human CC cell lines RBE, HCCC-9810 were purchased from the Type Culture Collection of

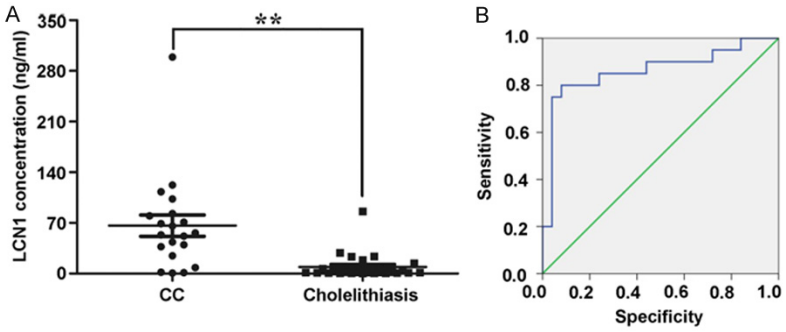

Figure 1. High expression of LCN1 in the bile from patients with cholangiocarcinoma. A. Comparison of the expression levels of LCN1 in the bile between cholangiocarcinoma group (n=20) and cholelithiasis group (n=25). B. Receiver operating characteristic (ROC) curve analysis of the correlation between expression of LCN1 protein in patients with cholangiocarcinoma and cholelithiasis.

the Chinese Academy of Sciences (Shanghai, China) and Huh28, HuCCT1, QBC939, HIBEpiC were kindly provided by 3D Medicines (Shanghai, China). Cells were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 100 μg/ml penicillin and 100 μg/ml streptomycin (Invitrogen, USA) in a 5% CO_2 atmosphere at 37°C.

Western blotting

Cells were lysed in lysis buffer, and total protein contents were determined by BCA Protein Assay Kit. A total of 30 μg of lysis solution was separated by the SDS-PAGE and transferred to the PVDF membrane. Blots were incubated with primary and secondary antibodies, and then detected by western bright ECL Plus (Advansta).

Immunohistochemistry (IHC)

The HCC tissue microarray containing 100 cases of intrahepatic cholangiocarcinoma, 27 cases of extrahepatic cholangiocarcinoma samples was purchased from Shanghai Outdo Biotech (Shanghai, China). Briefly, sections were deparaffinized in xylene, rehydrated in graded alcohol, immersed in 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity, and antigen retrieved by pressure cooking for 3 minutes in Tris/EDTA (pH= 8.0). LCN1 was detected by rabbit anti-LCN1 antibody (1:150, Abcam, ab128040) at 4°C overnight. After incubation with a secondary antibody for 30 minutes, the specimens were stained with DAB (3,3-diaminobenzidine), and then the nuclei were counterstained with hematoxylin.

Staining results were evaluated independently by two pathologists without prior knowledge of clinicopathologic data. Staining intensity was scored as follows: 0, no staining; 1+, mild staining; 2+, moderate staining; 3+, intense staining. The area of staining was evaluated as follows: 0, no staining of cells in any microscopic field; 1+, <30% of tissue stained positive; 2+, between 30% and 60% of tissue stained positive; 3+, >60% of tissue stained positive. LCN1 expression was evaluated by combining the staining intensity and extension. If the final score was equal or bigger than six, the protein expression in the tumor was considered high; otherwise, the protein expression in the tumor was considered low.

Statistical analysis

Results are shown as mean ± standard deviation. Statistical analyses were performed using SPSS version 20.0 (IBM Corporation, Armonk, NY, USA). All experiments were repeated three times. Values were presented as the mean ± standard deviation. Student's t-test, one-way ANOVA and χ^2 analyses were performed to analyze variance. Survival analysis was conducted using the Kaplan-Meier method, and the comparison of survival curves between groups was done with the log-rank test. *P*-value <0.05 were considered as significant.

Results

LC-MS/MS identified LCN1 expression was higher in the bile from patients from patients with CC than cholelithiasis

Bile from patients with cholangiocarcinoma and cholelithiasis was analyzed by high performance reverse liquid chromatography (RPLC) and liquid ion trap mass spectrometer (LC-MS/MS) technology combined with construction of complete protein expression differences in

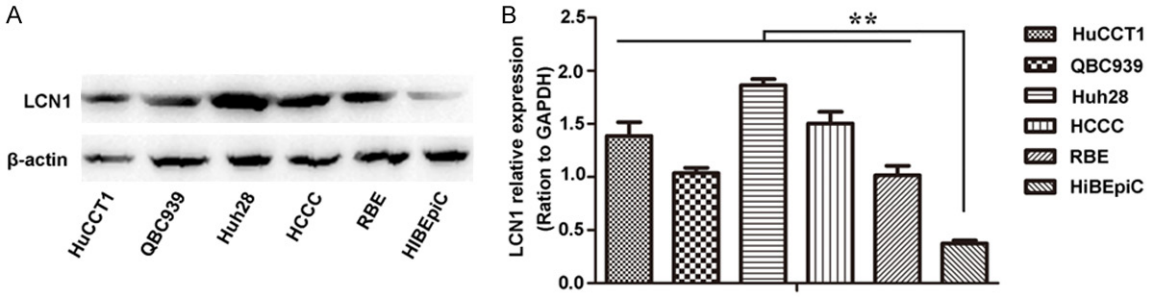

Figure 2. LCN1 expression is higher in cholangiocarcinoma cells than normal cholangiocytes. A. The expression of LCN1 was measured by western blotting in HIBEPIC, RBE, HCCC-9018, HUH28, HUCCT1, QBC939 cell lines. B. Relative LCN1 expression in different cell lines.

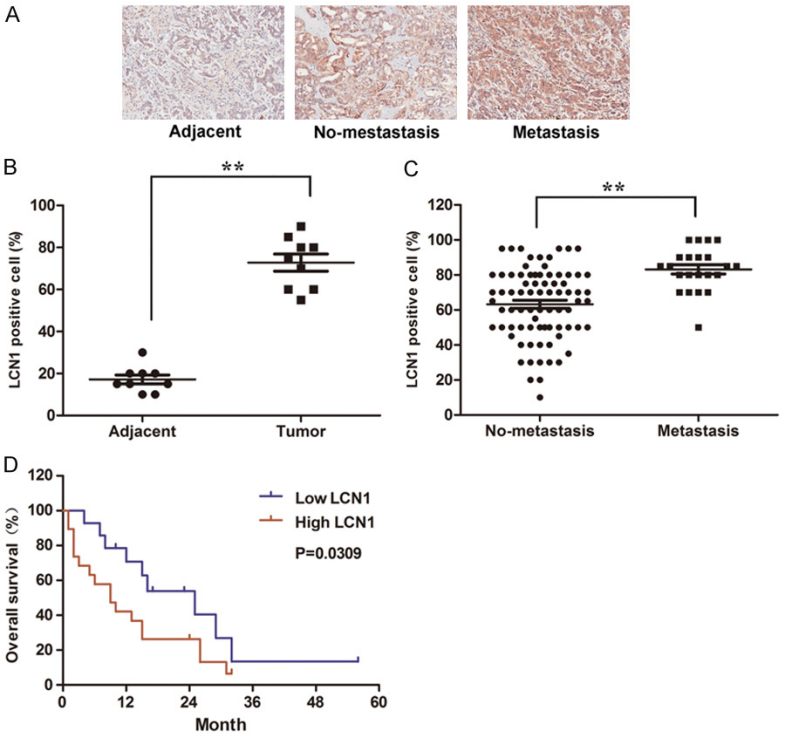

Figure 3. LCN1 is highly expressed in CCs, and correlates with distant metastasis and patient survival. A. LCN1 expression was analyzed by IHC analysis of paired CC and corresponding adjacent normal tissues. Representative results are shown. B. Comparison of the relative expression levels of LCN1 between CC and adjacent normal tissues. C. The association between LCN1 in CC as determined by IHC and survival time as analyzed by Kaplan-Meier survival analysis. D. Association between LCN1 expression in CC tissues and the survival time of selected patients was analyzed with Kaplan-Meier survival analysis.

human bile. RPLC was performed on bile samples from 15 CC and 10 cholangitis patients by HPLC. In total, RPLC-MS/MS detected 1985 proteins, found a total of 285 differentially expressed proteins, 198 of which were in the bile from CC patients with significantly higher expression, 87 proteins were in the bile of patients with low expression.

Moreover, using the STRING database of these proteins for bioinformatics analysis of combined disease the current progress of the study, selected LCN1 as a potential biomarker. These results suggest that the successfully identified differentially expressed proteins were derived from the bile.

LCN1 is highly expressed in the bile from CC, CC cells and CC tissues

To determine whether LCN1 expression correlated with development and progression of CC, we examined LCN1 expression in the bile from 20 cases with CC and 25 cases with cholelithiasis by ELISA. Compared with the cholelithiasis group, bile from CC patients were characterized by overexpressed levels of LCN1 (**Figure 1A**). Moreover, receiver operating characteristic (ROC) curve analysis revealed that LCN1 expression in the bile was statistically significant for differentiating patients with CC from cholelithiasis (AUC= 0.862, P<0.001) (**Figure 1B**).

Expression of LCN1 in HIBEPIC, RBE, HCCC-9018, HUH28, HUCCT1, QBC939 cell lines was next determined. LCN1 expression was remarkably upregulated in CC cells than cholangio-

Table 1. LCN1 staining and clinicaopathologic characteristics of CC patients

Variables	Lcn1 staining		Total	*p*
	Low	High		
Age (y)				
≤50	8 (11%)	13 (23%)	21	0.093
>50	63 (89%)	43 (77%)	106	
Sex				
Male	44 (62%)	36 (64%)	80	0.468
Female	27 (38%)	20 (36%)	47	
Tumor diameter				
≤5	22 (31%)	28 (50%)	50	0.023
>5	49 (69%)	28 (50%)	77	
Metastasis				
Absent	23 (32%)	9 (16%)	32	0.028
Present	48 (68%)	47 (84%)	95	
AJCC stage				
I+II	51 (72%)	49 (88%)	100	0.017
III+IV	20 (28%)	7 (13%)	27	

cytes (**Figure 2A**, **2B**). These findings indicated that LCN1 might play roles in CC development.

LCN1 is highly expressed in CCs, and correlates with distant metastasis and patient survival

LCN1 expression in CC samples by IHC was determined. The level of LCN1 protein was markedly higher in CC tissues than in adjacent normal tissues (**Figure 3A**, **3B**). LCN1 expression was also significantly correlated with tumor size and distant metastasis. No statistical connections were found between LCN1 expression and other clinicopathological parameters, such as patient age, gender (**Table 1**). To further assess the relevance of LCN1 expression in CC, the association between LCN1 expression and survival time was assessed in CC by Kaplan-Meier survival analysis (**Figure 3C**). The median overall survival time of the high LCN1 expression group was significantly shorter than that of the low LCN1 expression group (**Figure 3D**, P=0.0309). These results indicate that LCN1 may have a functional role in the aggressive behavior of HCCs.

Discussion

Chronic inflammation is a major contributor to carcinogenesis. In this study, we show that expression of LCN1 in tissue, cells, and bile of cholangiocarcinoma is higher than those in benign tissues. Therefore, our study is the first to report that LCN1 is an important tumor maker, which can distinguish CC from cholelithiasis, and may play an important role in the development of CC. These results suggest an oncogenic role of LCN1 in CC.

LCN1, first isolated from the lacrimal and lingual salivary glands as a highly produced protein, is a lipocalin member produced by a number of secreting glands and tissues. LCN1 is known to bind a variety of lipophilic compounds, including fatty acids, fatty alcohols, cholesterol, retinol, retinoic acid, phosphatidylcholine, and arachidonic acid and its peroxidation products [4, 6]. Although LCN1 is mainly secreted by the lacrimal glands and tongue salivary glands, it also expressed in several other secretory tissues such as the prostate, mucosal glands of the tracheobronchial tree, the nasal mucosa and sweat glands [7-9]. LCN1 is also one of the most important components of human tears. LCN1 increases the surface tension of the liquid, and distributes the tears evenly into the eyeball by binding to the lipid components of the tears [12]. Nevertheless, there is still emerging evidence supporting its major function for tissue and cell protection as a potentially harmful affinity for molecules [10]. High levels of LCN1 are found in the bronchoalveolar fluid from smokers than in nonsmokers [13]. Consistent with these observations, the promoter region of LCN1 contains several regulatory elements present in the gene encoding the acute phase protein [14]. LCN1 is involved in the innate immune response against bacterial and fungal infections, and it inhibits the in vitro-induced oxidation of lipid peroxidation products [10]. Lipocalin-1 interacting membrane receptor (LIMR), which interacts with LCN1, is essential for cellular internalization of LCN1 [15]. Recently, it has been reported that LCN1 protein and its receptor protein are involved in tumor cell metastasis and invasion. Anil B. Mukherjee found Lip-1R-hUG interaction on Lip-1R transfected HTB-81 cells rendered them fully responsive to hUG-mediated inhibition of migration and invasion [16]. Moreover, cDNA encoding a receptor for LCN1 has been characterized and antisense-mediated suppression of this receptor inhibits internalization of the LCN1 by NT2 cells [15]. However, whether LCN1 participant in tumorigenesis and the underlying mechanism is still largely unknown.

The dismal prognosis of cholangiocarcinoma is at least partially attributable to the lack of effective and convenient means of early diagnosis. Our studies show that LCN1 is significantly higher in CC than adjacent no-carcinoma tissue, indicating that LCN1 expression level has the potential to distinguish malignant from benign lesions origin from the bile duct. To investigate the diagnostic value of LCN1, CC cells and tissue microarray for cholangiocarcinoma were used in the following study. Our data showed that LCN1 expression level was significantly higher in tumor tissues than in normal tissues, and was correlated with tumor size, metastasis, clinical stage, and OS of CC patients, suggesting its potential as a diagnostic biomarker for CC. Therefore, we propose that LCN1 might play an important role in the process of development in CC.

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Disclosure of conflict of interest

None.

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