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Expression and Prognostic Value of PRL-3 in Human Intrahepatic Cholangiocarcinoma

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Abstract Phosphatase of regenerating liver (PRL)-3 is involved in the metastasis of various tumors, but the expression of PRL-3 and its possible role in primary intrahepatic cholangiocarcinoma (ICC) has not been reported yet. In this study, we assessed the expression levels of PRL-3 by immunohistochemistry in 102 primary ICC samples, 62 matched lymph node metastases (LNM) and 102 adjacent normal liver tissues. Then we investigated the relationship between PRL-3 expression and clinicopathologic factors. Survival analysis was performed to determine the prognostic significance of PRL-3 expression in ICC. Immunochemistry results suggested PRL-3 expression was negative or weak in non-neoplastic intrahepatic bile ducts of adjacent liver tissue. In primary lesion and LNM high PRL-3 expression was frequently detected. Furthermore, the rate of high PRL-3 expression in LNM was higher than that in primary lesion (80.6% *vs.* 47.1%, $P<0.05$). High expression of PRL-3 in primary tumors was significantly associated with TNM ($P<0.001$), T stage ($P<0.001$), vascular invasion ($P=0.002$), and LNM ($P<0.001$). Survival analysis results with Kaplan-Meier method and Cox proportional hazard model indicated high expression of PRL-3 was correlated with decreased overall survival and was an independent prognostic marker of overall survival. Thus, our results suggested high expression of PRL-3 was correlated with progression and metastasis of ICC and indicated negative prognostic impact. PRL-3 might serve as a novel prognostic marker for patients with ICC.

Keywords Intrahepatic cholangiocarcinoma · PRL-3 · Lymph node metastasis · Immunochemistry · Prognosis

Abbreviations

PRL-3	phosphatase of regenerating liver-3
ICC	intrahepatic cholangiocarcinoma
LNM	lymph node metastasis
RR	relative risk
CI	confidence interval

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Introduction

Intrahepatic cholangiocarcinoma (ICC), arising from cholangiocytes of intrahepatic bile ducts, is a rare primary malignant liver cancer compared with hepatocellular carcinoma (HCC). It has been reported that ICC accounts for only about 10% of primary liver cancers. However, in recent years the incidence of ICC has increased worldwide [1–5]. The ICC is considered to be a highly fatal carcinoma because of early invasion, widespread metastasis, and the lack of an effective therapy [6, 7]. Despite improved diagnostic and operative techniques, the prognosis of ICC remains poor [1, 2, 8]. In contrast to HCC, early lymphatic

spread tends to occur in ICC. Moreover, lymph node status is an important prognostic factor for patients with ICC [9, 10]. Although several molecules and histological features have been reported to be associated with prognosis and metastasis of ICC [11, 12], more valuable biomarkers are needed to predict the clinical outcome of patients with ICC.

Protein tyrosine phosphatases, a subgroup of the protein phosphatase superfamily, play key roles in regulating diverse proteins that participate essentially in every aspect of cellular physiological and pathogenic processes [13–15]. Phosphatase of regenerating liver (PRL-1, -2, and -3) makes up a novel class of non-classical protein tyrosine phosphatases with a CAAX sequences at the C-terminal for prenylation [16]. All the three members are proteins of about 20-kD and share 76%–87% amino acid sequence identity. The first PRL to be identified, PRL-1, was originally discovered as an immediate early gene whose expression is induced in mitogen-stimulated cells and regenerating liver (thus named phosphates of regenerating liver-1) [17, 18]. Two other PRLs, PRL-2 and PRL-3, were subsequently identified by amino acid sequence homology [16].

Considerable evidence suggests PRL phosphatases, especially PRL-3, might play significant roles in growth regulation, proliferation, promoting cell motility, invasion and metastasis [19–21]. Distinct from the wider expression of PRL-1 and PRL-2, among normal human adult tissues,

Table 1 Relationship between PRL-3 expression in primary intrahepatic cholangiocarcinoma and clinicopathologic factors

	PRL-3					χ^2	*P*
	Cases	High		Low			
	n	n	%	n	%		
Total	102	48	47.1	54	52.9		
Sex						1.71	0.191
Male	70	36	51.4	34	48.6		
Female	32	12	37.5	20	62.6		
Age							
≥60	72	32	44.4	40	55.6	0.672	0.4
<60	30	16	53.3	14	46.7		
Histology						2.469	0.291
Well	14	4	28.6	10	71.4		
Moderate	66	32	48.5	34	51.5		
Poorly	22	12	54.5	10	45.5		
Tumor size						0.153	0.696
≤4.0 cm	36	16	44.4	20	55.6		
>4.0 cm	66	32	48.5	34	51.5		
Vascular invasion						9.294	0.002
Present	56	34	60.7	22	39.3		
Absent	46	14	30.4	32	69.6		
CA199						5.399	0.02
≤35KU/L	42	14	33.3	28	66.7		
>35KU/L	60	34	56.7	26	43.3		
Lymphoid node metastasis						27.15	<0.001
No	40	10	25.0	30	75.0		
Yes	62	38	61.3	24	38.7		
TNM stage						20.41	<0.001
land II	20	2	10.0	18	90.0		
III	46	20	43.5	26	56.5		
IV	36	26	72.2	10	27.8		
T stage						18.62	<0.001
T1-T2	46	16	34.8	30	65.2		
T3	26	8	30.8	18	69.2		
T4	30	24	80.0	6	20.0		

PRL-3 is expressed predominantly in the heart and skeletal muscle cells with lower expression in the pancreas [20]. Since PRL-3 was first linked to metastasis from genome-wide transcriptional analysis of colorectal cancer samples, it has gained many interests as a metastasis-related gene [22]. Stable expression of wild-type PRL-3 could enhance cell motility and invasive ability in *vitro*, and promote metastasis in mouse model systems [21, 23]. Oppositely, down-regulation of PRL-3 expression by interfering RNA could attenuate the ability of motility and invasion in *vitro* and suppress metastasis in nude mice [24, 25]. High expression of PRL-3 has been reported in a variety of cancer cell lines and tissues [26], including gastric cancer [27, 28], breast cancer [29] and ovarian cancer [30, 31]. Furthermore, PRL-3 was demonstrated to be a useful indicator for tumor recurrence and patient outcome in several human cancers [28, 29, 31].

However, up to now, the PRL-3 expression level in ICC and matched LNM has not been determined; and its clinical significance in ICC is unclear. In this study, we determined the expression of PRL-3 by immunohistochemistry assay and investigated the relationship between PRL-3 expression and clinicopathologic features. Survival analysis was employed to evaluate the impact of high PRL-3 expression on the prognosis of patients with ICC.

Materials and Methods

Patients and Tissues Samples

Tissues of 102 primary intrahepatic cholangiocarcinoma, 62 matched metastatic lymph nodes, 102 adjacent non-cancerous liver tissue containing normal intrahepatic bile ducts (at least 5 cm distant from the tumor edge) were obtained from the Department of Pathology, Shandong Provincial Hospital. Each sample had been fixed in formalin, routinely processed, and embedded in paraffin. The main clinical and pathologic variables of the 102 ICC patients are shown in Table 1. They consisted of 70 males and 32 females with an average age of 55 years (ranging from 36 to 72). All cases reviewed were diagnosed histopathologically and graded according to the current WHO criteria (WHO histological classification of tumors of the liver and intrahepatic bile ducts) [32]. Informed consent was obtained from all patients, and no patients received any type of therapy before surgery.

Immunohistochemical Staining

The expression levels of PRL-3 in tissue sections were analyzed by immunohistochemistry, performed using the streptavidin–biotin–peroxidase method with labeled

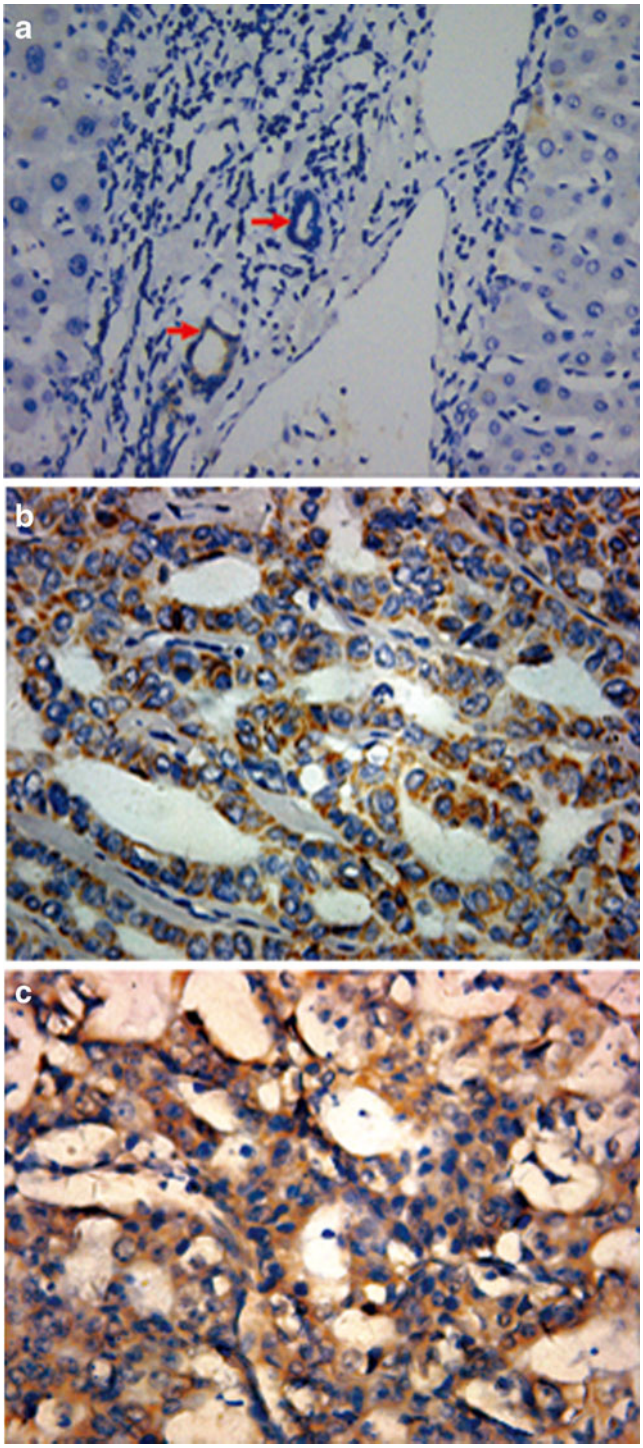

Fig. 1 Expression of PRL-3 in normal intrahepatic bile ducts, primary tumors and lymph node metastases was measured by immunochemistry. Representative results of PRL-3 staining were shown. **a** The expression of PRL-3 is negative or weak in normal bile ducts (red arrows, original magnification, 200×) **b** intrahepatic cholangiocarcinoma tissue with high PRL-3 expression (original magnification, 400×) **c** Lymph node metastasis with high PRL-3 expression (original magnification, 400×)

streptavidin–biotin. Briefly, four micrometer-thick sections obtained from formalin-fixed, paraffin-embedded tissue blocks were baked at 50°C–60°C for at least 2 h, deparaffinized with xylene and rehydrated through a grade alcohol series. For antigen retrieval, the slides were placed in a glass box filled with 10 mmol/l citrate buffer (pH 6.0) and were boiled for 15 min at 100°C. Then the sections was allowed to cool in the box at room temperature and placed in 3% hydrogen peroxide solutions to inhibit endogenous peroxidase activity. 1% goat serum was applied to sections to block nonspecific binding. The primary monoclonal mouse antibody, anti-PRL-3 (1:100 dilution; R&D Systems, Boston, MA) was incubated at 37°C for 2 h (or overnight at 4°C in a moist chamber). After being washed, sections were incubated at 37°C with biotinylated goat anti-mouse IgG for 30 min and streptavidin conjugated to horseradish peroxidase for 30 min (SP-9000 Histostain™–Plus Kit; Zymed Laboratories, San Francisco, CA, USA). Signal was detected by a standard streptavidin immunoperoxidase reaction, followed by chromagen detection with diaminobenzidine for 10 min at room temperature. The sections were then counterstained with hematoxylin. For negative control, the primary antibody was replaced by normal mouse serum. The staining in the cytoplasm and the cytoplasmic membrane was evaluated semiquantitatively. The frequency of PRL-3 positive cells was scored on the basis of the percentage of positive cells as 0%=negative; 1–25% = +1; 26–50% = +2; and >50% = +3. The intensity of PRL-3 expression was scored as weak=1, moderate=2 and strong=3. The average PRL-3 expression of each section was calculated as intensity multiplied by frequency and classified as low (≤2) or high (>2) [33]. All of the sections were scored twice to confirm the reproducibility of the results.

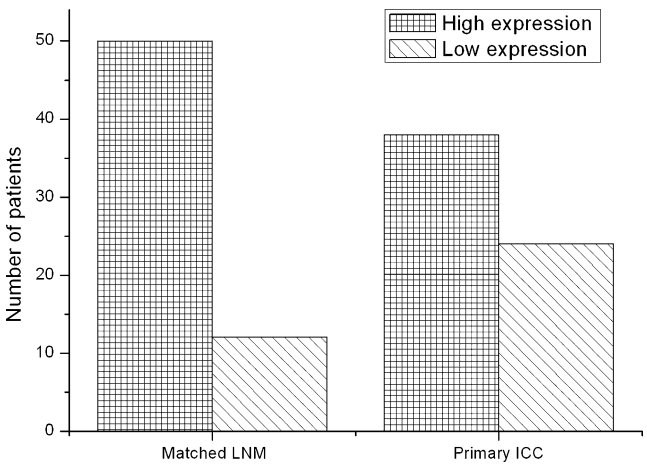

Fig. 2 High expression of PRL-3 was more frequently detected in matched lymph node metastasis than in primary cancer ($p<0.05$)

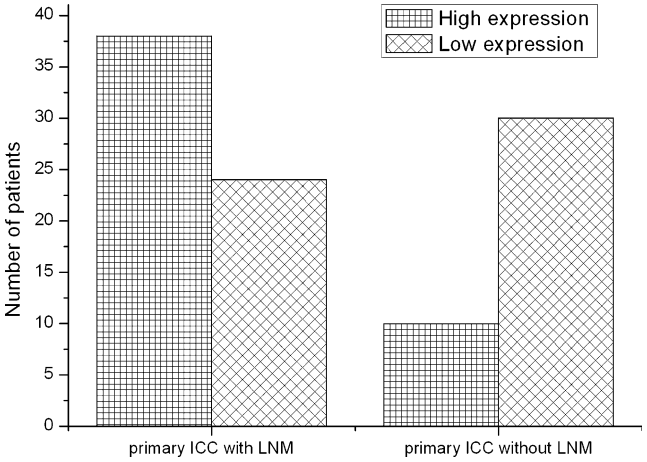

Fig. 3 High expression of PRL-3 was more frequently detected in the primary cancer with lymph node metastasis than that without lymph node metastasis ($p<0.001$)

Prognosis Analysis

All 102 patients with ICC had been followed up. The follow-up period ranged from 9 to 60 months with a median follow-up period of 30.5 months. Unfortunately, 7 patients (6.9 %) lost the follow-up. Log-rank test was performed to compare overall survival rates between patients with high PRL-3 expression and those with low PRL-3 expression in the primary lesion.

Statistical Analysis

The analyses of data were performed using the software package SPSS15.0 for Windows. χ^2 test was used to

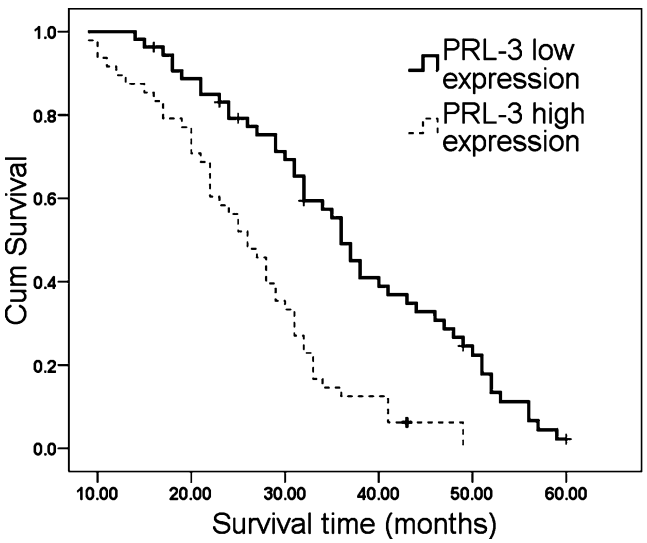

Fig. 4 Survival curves using Kaplan–Meier method. Survival curves showed patients with high PRL-3 expression in the primary tumors presented lower overall survival rate than those with low expression ($p<0.001$)

Table 2 Univariate analysis of prognostic factors for overall survival

Variable	χ^2	*P* value
Age	0.086	0.769
Sex	0.002	0.961
Tumor Size	0.391	0.532
Vascular invasion	1.994	0.158
CA199	5.223	0.022
LN metastasis	16.422	<0.001
TNM stage	14.190	0.003
T stage	7.775	0.051
PRL-3 expression level	21.413	<0.001

determine the statistical significance of rate difference and assess the association between PRL-3 expression and clinicopathologic characteristics. Log-rank test according to Kaplan–Meier survival analysis approach was used to compare overall survival rates of patients. Cox simultaneous proportional hazards models were constructed for multivariate analyses of survival. Significance was set at $P<0.05$.

Results

Immunohistochemical Detection of PRL-3 in Primary ICC, Matched Metastatic Lymph Nodes and Adjacent Non-cancerous Intrahepatic Bile Ducts

The expression and localization of PRL-3 was measured by the method of immunochemistry in 102 primary ICC, 62 matched metastatic lymph nodes. Meanwhile, 102 cases matched adjacent non-cancerous liver tissues containing normal intrahepatic bile ducts were examined. Representative results of PRL-3 staining are shown in Fig. 1. We found that PRL-3 expression was negative or low in adjacent non-cancerous intrahepatic bile ducts. But in cholangiocarcinoma tissues and LNM, the rate of high PRL-3 expression was 47.1% (48/102) and 80.6%(50/62) respectively. The PRL-3 expression in primary ICC and its LNM was significantly higher than that in normal intrahepatic bile ducts ($P<0.05$). To more precisely examine PRL-3 expression, we compared the PRL-3 expression in cholangiocarcinoma accompanying LNM with that in matched metastatic lymph node (Fig. 2). In 62 primary tumor lesions, the proportion of high PRL-3 expression was 61.3% (38/62). While in their matched LNM, the proportion was 80.6% (50/62).The rate of high expression of PRL-3 was significantly higher in LNM compared with matched primary ICC ($P<0.05$). We also assessed the PRL-3 expression of 40 primary cancer lesions without LNM, and the result showed that 25% of them was with high PRL-3 expressions (Fig. 3). Statistic analysis showed that the expression of PRL-3 was more frequently detected in the primary lesion with LNM than that without LNM ($P<0.001$).

PRL-3 Expression and Clinicopathologic Characteristics

The relationship between PRL-3 expression and clinicopathologic factors of ICC was investigated, and the results were summarized in Table 1. We found that among patients with high PRL-3 expression in primary tumor, 70.8%(34/48) developed vascular invasion, 79.2%(38/48) accompanied LNM, 54.2% (26/48) had TNM stage IV tumors, and 66.7%(32/48) had T3 or T4 tumors. These results suggested expression of PRL-3 in primary tumors was significantly associated with progression and metastasis of tumors, such as TNM ($P<0.001$), T stage ($P<0.001$), vascular invasion ($P=0.002$), and LNM ($P<0.001$). Meanwhile, our results demonstrated that there was a significant correlation between high PRL-3 expression and high serum CA19-9 level ($P<0.05$). No significant correlation was observed between PRL-3 expression and age, gender, histology and tumor size.

Table 3 Multivariate analysis of overall survival

Variable	χ^2	*P* value	RR	95% CI for RR	
				lower	upper
Age	1.735	0.188	1.720	0.767	3.857
Sex	0.008	0.927	0.965	0.447	2.080
LN metastasis	1.599	0.206	1.498	0.801	2.804
CA199	2.669	0.102	1.492	0.923	2.411
TNM stage	3.466	0.325			
TNM(IIvs.I)	0.155	0.288	1.403	0.259	7.589
TNM(IIIvs.I)	1.128	0.230	2.366	0.483	11.587
TNM(IVvs.I)	1.439	0.206	2.542	0.554	11.675
PRL-3 expression level	5.007	0.024	1.886	1.086	3.277

PRL-3 Expression and Intrahepatic Cholangiocarcinoma Prognosis

The Kaplan-Meier survival curve and Cox proportional hazard model were used to determine the prognostic significance of PRL-3 expression in ICC. The Kaplan-Meier survival curve showed that patients with high level of PRL-3 in primary tumor tended to have shorter survival time than those with low level of PRL-3 (the median survival 26 months *vs.* 36 months). Log-rank test results suggested a significant difference in overall survival rates between the two group (Fig. 4; $P<0.001$). Univariate analysis also identified other significant prognostic factors besides PRL-3, such as LNM ($P<0.001$), CA19-9 ($P=0.02$) and TNM stage ($P=0.003$). In contrast, the association of patients' age, gender, T stage and tumor size with prognosis was not significant in univariate analysis (Table 2).

In a multivariate analysis, significant factors in the univariate analysis of overall survival (expression level of PRL-3, TNM stage, LNM, and CA19-9 serum level) and adjusting factors (gender, age) were estimated. The result showed that PRL-3 expression was an independent prognostic marker of overall survival (RR 1.886, $P=0.024$, Table 3). All of these indicated PRL-3 might be a valuable prognostic marker for patients with ICC.

Discussion

PRL-3, also known as PTP4A3, is a newly identified metastasis-related gene. Increasing evidences have demonstrated high expression of PRL-3 might be a key alteration contributing to the invasion and metastasis of tumor cells. Recently, PRL-3 was also proved to be associated with prognosis in several kinds of tumors [28, 29, 31, 34]. In the present study, we firstly examined PRL-3 expression in primary ICC, matched metastatic lymph nodes, adjacent normal intrahepatic bile ducts by immunochemistry assays. We found that the expression of PRL-3 was obviously higher in LNM than that in primary ICC. Moreover, high expression of PRL-3 was more frequently discovered in primary ICC with LNM than those without LNM. These findings were consistent with the report of gastric cancer and colorectal cancer [28, 34]. Next, we investigated the relationship of PRL-3 expression and clinicopathologic characteristics. A significant association was found between PRL-3 expression and vascular invasion, LNM, TNM stage and T stage. This suggested that high level of PRL-3 was associated with aggressiveness of ICC. In addition, we also detected that PRL-3 expression was correlated with the serum level of CA19-9. Serum CA19-9 levels was helpful to early diagnosis and prognosis of cholangiocarcinoma [35–37]. Thus, we raise a hypothesis that combination detection the two markers might be more accurate to predict the outcome of patients with ICC. To confirm the hypothesis, further researches are needed. Inconsistent with previous report in gastric cancer [28], no significant correlation was observed between PRL-3 expression and tumor size in our study.

Previous studies have demonstrated high level of PRL-3 in primary cancer was correlated with the prognosis of tumor patients. In our study, univariate survival analysis data showed that the median survival of patients with a high level of PRL-3 in primary tumor was shorter than those with a low level of PRL-3, and the patients with highly expressing PRL-3 in primary tumors tended to have a lower overall survival rate compared with those with low PRL-3 expression. Multivariate analysis result suggested PRL-3 expression level in primary lesion was an independent prognostic marker for overall survival after adjusting for other prognostic factors. Thus, our survival analysis results indicated that PRL-3 may serve as a potential prognostic marker in ICC.

Taking together, our study suggests that overexpressed PRL-3 was correlated with progression and metastasis in ICC. PRL-3 is considered to be an independent prognostic factor for overall survival in patients with ICC. Nevertheless, the substrates and the signaling pathways of PRL-3 are not well known, and specific inhibitors are not available, further investigations are needed to illuminate the functions of PRL-3 in metastasis and to develop a new therapy.

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