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Overexpression of LAPTM4B-35 closely correlated with clinicopathological features and post-resectional survival of gallbladder carcinoma

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ABSTRACT

Gallbladder carcinoma (GBC) is a malignancy with dismal prognosis and unclear gene expression profile. We aimed to first present the expression of LAPTM4B-35, one product of a cancer associated gene recently cloned in hepatocellular carcinoma (HCC), and its correlation with clinicopathological features and prognosis of GBC. Immunohistochemical detection of LAPTM4B-35 was performed on samples from 75 patients with GBC. LAPTM4B-35 protein was overexpressed in 57 patients (76%) with GBC. The staining scores were significantly related to histology type, lymph node involvement, distant metastasis, Nevin staging and differentiation of GBC ($P < 0.05$). Univariate analysis revealed that overall or disease-free survival of patients was inversely associated with its staining scores ($P < 0.001$). Multivariate analysis showed that LAPTM4B-35 staining score was an independent prognostic marker for both overall and disease-free post-resectional survival of GBC ($P = 0.004$ and 0.027, respectively). LAPTM4B-35 overexpressed in a majority of GBCs and correlated with clinicopathological features and post-resectional survival.

1. Introduction

Gallbladder carcinoma (GBC) is a malignancy with extensively poor prognosis.[1,2] Although GBC used to be identified as a relatively uncommon tumour, its higher or increased incidence was reported in some populations,[3–6] including residents in some areas of China. Epidemiological data showed that incidence of GBC had a 140% increase in men and 126% in women in urban Shanghai.[6] Therefore, many studies focused on genes and their products involved in different stages of gallbladder carcinogenesis, such as p53,[7] K-ras,[8] β-catenin,[9] cyclin D1,[10] $p27^{Kip1}$,[11] mucins and cytokeratins.[12] Besides, some of them were shown to be of prognostic significance.[10,11] However, further comprehensive investigations and new clues were expected.

Recently, a novel gene, overexpressed in hepatocellular carcinoma (HCC) and designated by HUGO as *LAPTM4B* (lysosome-associated protein transmembrane-4 beta), was successfully cloned by fluorescence differential display, rapid amplification of cDNA ends (RACE) and RT-PCR.[13,14] It has been clarified that *LAPTM4B* gene is mapped to chromosome 8q22.1, with seven exons separated by six introns.[14] Interestingly, *LAPTM4B* gene encoded two proteins with different molecular weight, 35 kDa and 24 kDa.[15] Experiments revealed that expression of *LAPTM4B*, at both mRNA and protein levels, was highly upregulated in most specimens and inversely cor-

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related with differentiation of HCC.[14–16] What calls for special attention is that the main protein overexpressed in HCC is LAPTM4B-35.[15,16] Current data showed that *LAPTM4B* was also remarkably expressed in several cancers, such as breast cancer, lung cancer, gastric cancer, colon cancer, uterus cancer, ovary cancer, adrenocorticotrophin (ACTH)-secreting and non-functioning pituitary adenoma, but not commonly expressed in oesophageal and rectum cancers.[16–18] However, there has not been any evidence concerning its expression status in GBC, so far. Furthermore, clinicopathological relevance and significance of *LAPTM4B* overexpression in GBC remain unknown.

The present study aims to show expression of LAPTM4B-35 in GBC and to investigate its relationship to clinicopathological features and its impact on post-resectional survival.

2. Materials and methods

2.1. Patients

Matched cancerous tissues and adjacent non-cancerous gallbladder epithelia were obtained from 75 consecutive patients with GBC undergoing surgery in our institution from 1991 to 2002. There were 49 females and 26 males (median age, 62 years; range, 30–88 years). Histological type, lymph node involvement and differentiation grade for GBC were determined with routine pathological examination after surgery. Distant metastases were found by imaging examinations and confirmed during operation. All patients were staged according to Nevin's criteria.[19] Tumour size was defined as the largest dimension for solitary mass and the sum of the largest dimension of each mass for multiple masses. The clinicopathological features of patients are shown in Table 1. Fifty-one patients (68%) underwent radical resection (R0), whereas the other 24 patients (32%) underwent palliative resection (R1) because of distant metastasis (16 patients) and positive resection margin (eight patients). The Institutional Ethics Committee approval for this project was obtained.

2.2. Immunohistochemical staining

LAPTM4B-35 expression was detected using immunohistochemistry for paraffin-embedded cancerous and non-cancerous specimens obtained from 75 patients with GBC. Rabbit anti-human polyclonal antibody (LAPTM4B-$N_{1\text{-}99}$-pAb), specifically recognising LAPTM4B-35 (but not LAPTM4B-24) protein, was raised and kindly provided by Prof. Rou-Li Zhou, Department of Cell Biology, Peking University Health Science Centre, as the method reported previously.[16] In brief, 4 μm-thick sections, cut from paraffin-embedded tissue blocks, were mounted on adhesive-coated glass slides, deparaffinised in xylene, rehydrated in ethanol and treated with 3% hydrogen peroxide for 20 min to block endogenous peroxidase. Pretreatment was performed using 0.1% trypsin for 15 min to retrieve antigen. After washing in Tris buffer, slides were incubated for 70 min at room temperature with the primary antibody (dilution 1:20). Following washing three times in Tris

Table 1 – Relationship between LAPTM4B-35 expression and clinicopathological features of GBC

Variables	Patient number	Staining score of LAPTM4B-35				P[a]
		0	1	2	3	
Histology type						
AC	64	11	7	25	21	0.030
ASCC	5	3	1	1	0	
SCC	5	4	1	0	0	
SRCC	1	0	0	1	0	
Lymph node involvement						
Present	26	5	2	5	14	0.004
Absent	49	13	7	22	7	
Distant metastasis						
Present	16	2	1	3	10	0.009
Absent	59	16	8	24	11	
Nevin staging						
I	4	2	1	1	0	0.010
II	12	6	3	2	1	
III	15	4	4	2	5	
IV	9	2	1	4	2	
V	35	4	0	18	13	
Differentiation						
G1	24	12	3	6	3	0.017
G2	30	4	3	11	12	
G3	21	2	3	10	6	

LAPTM4B, lysosome-associated protein transmembrane-4 beta; GBC, gallbladder carcinoma; AC, adenocarcinoma; ASCC, adenosquamous cell carcinoma; SCC, squamous cell carcinoma; SRCC, signet-ring cell carcinoma; G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated.
a Chi-square test.

buffer, horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody for En Vision method (K4003, DAKO Corporation, Carpinteria, USA) was added for an incubation period of 45 min. Diaminobenzidine was used as a chromogen. Finally, slides were counterstained with haematoxylin. Pre-immune rabbit serum at the same dilution was used as the negative control. Two observers who were blinded to clinical and follow-up data (J. C. and Q.C. C.) evaluated staining results independently and co-observed for a consensus when they were divergent.

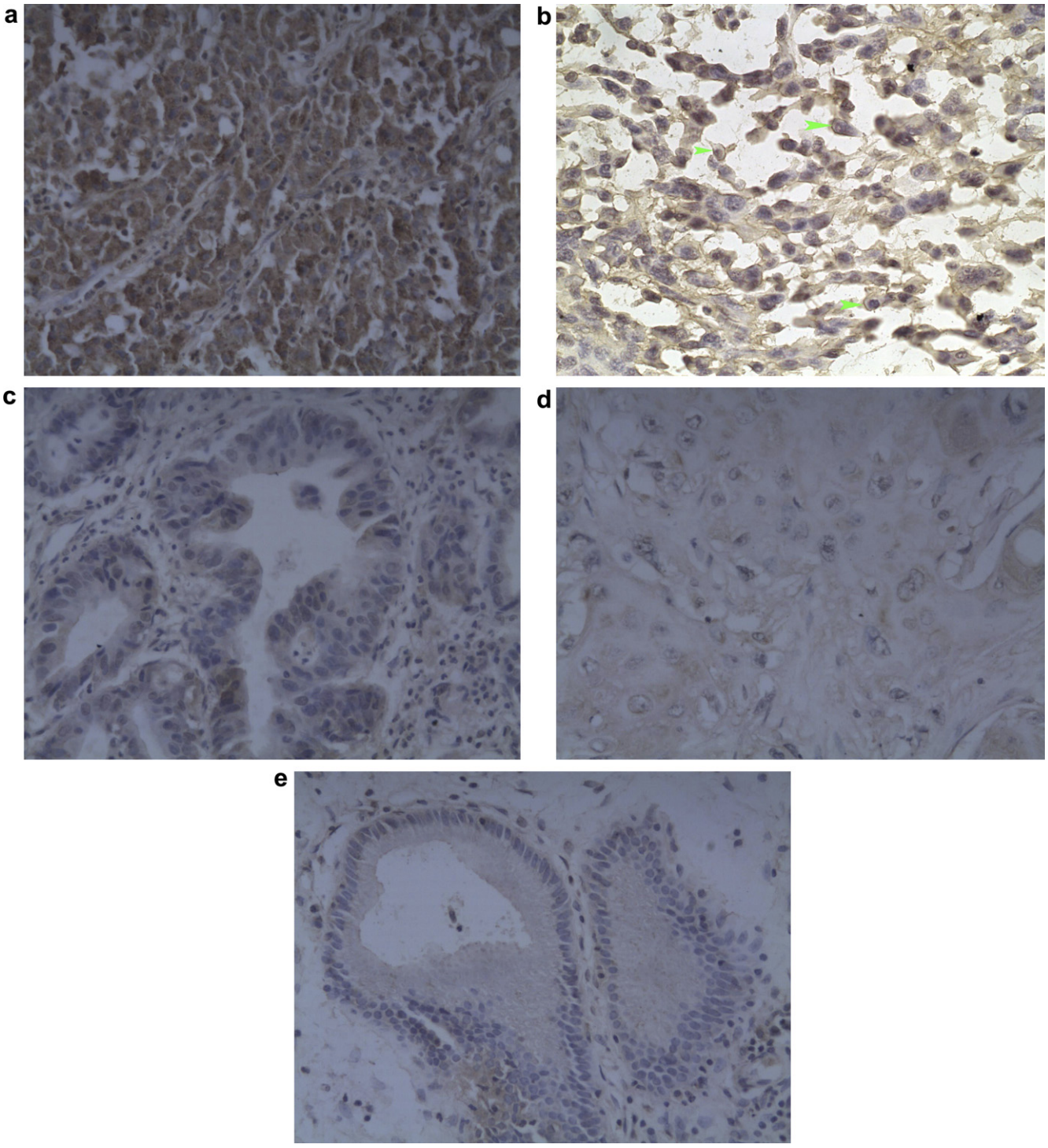

Fig. 1 – Expression of lysosome-associated protein transmembrane 4B-35 in gallbladder carcinoma. (a) Poorly differentiated adenocarcinoma (score 3, original magnification ×200); (b) poorly differentiated adenocarcinoma (positive staining of cell membrane, original magnification ×400); (c) well differentiated adenocarcinoma (score 1, original magnification ×200); (d) squamous cell carcinoma (score 1, original magnification ×200); (e) adjacent non-cancerous gallbladder epithelium (negative, original magnification ×200).

2.3. Staining evaluation

The brown colouration that located in cytoplasm or cell membrane, thus conforming to the structural characteristics of the protein, or transmembrane location, was defined as the positive signal. The cancerous staining extent of LAPTM4B-35 was classified in four grades, no staining: positive signal in 0 to 10% of cancer cells; 1+: positive signal in 11 to 25% of cancer cells; 2+: positive signal in 26 to 75% of cancer cells; 3+: positive signal in ⩾76% of cancer cells. In addition, its positive staining intensity was divided into three subgroups (faint, moderate and strong staining). Integrating these two aspects, overall results were expressed as the staining score, score 0: extent of no staining grade with intensity not stronger than the faint; score 1: extent of 1+ grade with intensity not stronger than the moderate or extent of 2+ grade with intensity not stronger than the faint; score 2: extent of 2+ grade with intensity of the moderate and the strong or extent of 3+ grade with intensity of the moderate; score 3: extent of 3+ grade with intensity of the strong. Samples were designated as overexpression of LAPTM4B-35 when they simultaneously met two criteria as below, (1) staining score in cancerous tissue was at least 1; (2) stronger staining intensity in cancerous tissue than in adjacent non-cancerous epithelia.

2.4. Follow-up

Sixty-nine patients were enrolled in our follow-up system with follow-up time ranging from 4 to 75 months (median, 20 months), whereas the other six patients censored postoperatively. Up to December 2004, 32 out of 69 patients died of progression of GBC (30 patients) and other conditions (one case of lung cancer and one of acute cardiac infarction), 23 patients censored during the follow-up period, and the remaining 14 patients were alive.

2.5. Statistical analysis

The Chi-square test was used to clarify differences of categorical variables. Overall or disease-free survival was analysed by the Kaplan–Meier method. Their differences were verified by log-rank test. Cox regression (Proportional hazard model) was adopted for multivariate analysis of prognostic predictors. Twenty-two patients who underwent palliative resection and follow-up were excluded from disease-free survival analysis. Statistical software package SPSS11.5 (SPSS Inc., Chicago, Ill) was employed for all analyses. Statistically significant P value was defined as <0.05.

3. Results

3.1. Expression of LAPTM4B-35 and its relationship with clinicopathological features of GBC

Overexpression of LAPTM4B-35 was found in specimens from 57 patients (76%) with GBC, according to abovementioned criteria (Fig. 1). Nine, 27 and 21 overexpressed cancerous samples were exhibited with staining scores 1, 2 and 3, respectively. The staining score significantly correlated with histology type, lymph node involvement, distant metastasis, Nevin staging and differentiation of GBC ($P < 0.05$; Table 1), but not with age, sex, tumour size and serum level of tumour antigen markers ($P > 0.05$, data not shown).

3.2. The impact of LAPTM4B-35 expression on overall and disease-free post-resectional survival of GBC

After excluding censored patients (4/57 of patients overexpressing LAPTM4B-35 versus 2/18 of patients not overexpressing LAPTM4B-35, $P = 0.952$), the impact of both clinicopathological variables and LAPTM4B-35 expression on

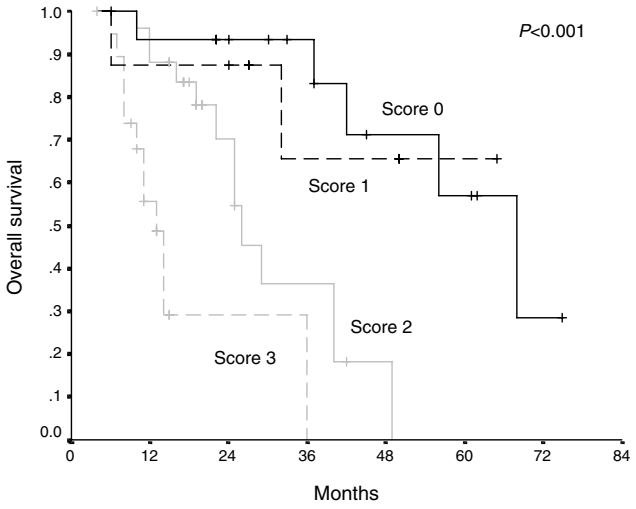

Fig. 2 – Impact of LAPTM4B-35 expression status on overall post-resectional survival of 69 patients with gallbladder carcinoma. Score 0, black solid line ($n = 16$). Score 1, black dashed line ($n = 8$). Score 2, gray solid line ($n = 26$). Score 3, gray dashed line ($n = 19$).

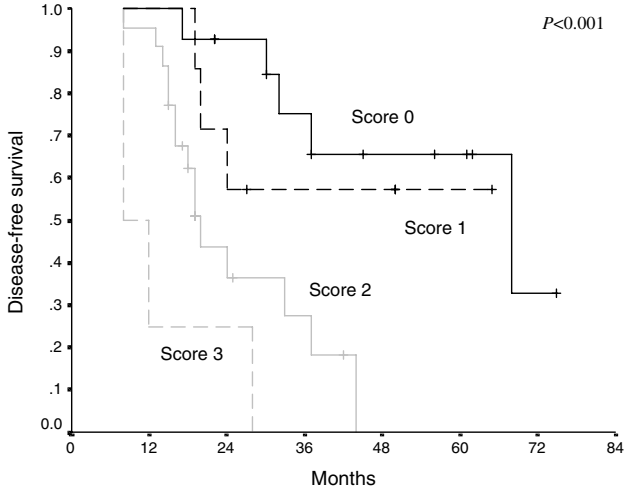

Fig. 3 – Impact of LAPTM4B-35 expression status on disease-free survival of 47 patients with gallbladder carcinoma after radical resection. Score 0, black solid line ($n = 14$). Score 1, black dashed line ($n = 7$). Score 2, gray solid line ($n = 22$). Score 3, gray dashed line ($n = 4$).

overall and disease-free post-resectional survival was determined using the Kaplan–Meier method and log-rank test. The staining scores of LAPTM4B-35 were inversely related to overall or disease-free post-resectional survival ($P < 0.001$; Figs. 2 and 3, and Table 2). Patients with staining scores of 0, 1, 2, and 3 had overall survival at 5-years of 56.9%, 65.6%, 0% and 0%, and disease-free survival at 5-years of 65.7%, 57.1%, 0% and 0%, respectively. These findings indicated that the staining score of LAPTM4B-35, similar to histology type, tumour size, lymph node involvement, Nevin staging and differentiation, etc., was of predicting significance for either overall or disease-free survival of GBC ($P < 0.05$; Table 2). Meanwhile, sex, age and serum antigens were not of value for both (Table 2). Multivariate analysis, that enrolled aforementioned significant parameters, indicated that LAPTM4B-35 staining score was an independent prognostic factor for both overall and disease-free post-resectional survival of GBC ($P < 0.05$; Table 3), together with some conventional clin-

Table 2 – Factors affecting overall and disease-free survival of patients with GBC

Variables	OS				DFS			
	n	Median ± SE	95%CI	P[a]	*n*	Median ± SE	95%CI	P[a]
Histology type								
AC	62	40 ± 3	33–47	0.006	44	33 ± 5	24–42	0.002
ASCC	4	13 ± 4	4–22		2	14 ± 1	12–16	
SCC	3	10 ± 3	4–16		1	17		
Tumour size								
>2 cm	50	29 ± 4	21–37	<0.001	32	20 ± 3	15–25	<0.001
⩽2 cm	19	68 ± 9	50–86		15	68 ± 17	34–102	
Lymph node involvement								
Present	23	22 ± 5	11–33	<0.001	9	16 ± 2	12–20	<0.001
Absent	46	42 ± 6	30–54		38	37 ± 4	29–45	
Nevin staging								
I	3	72 ± 2	67–76	<0.001	3	72 ± 2	67–76	<0.001
II	12	56 ± 9	39–73		12	51 ± 4	43–58	
III	13	37 ± 6	25–49		12	32 ± 4	25–39	
IV	7	22 ± 4	13–31		6	16 ± 3	10–22	
V	34	19 ± 5	9–29		14	16 ± 2	13–19	
Serum CEA level								
>5 ng/ml	28	32 ± 8	17–47	0.062	20	20 ± 8	14–26	0.040
⩽5 ng/ml	41	42 ± 8	26–58		27	44 ± 11	22–66	
Serum CA19-9 level								
>37 U/ml	45	29 ± 4	21–37	0.036	27	24 ± 3	19–29	0.058
⩽37 U/ml	24	49 ± 9	30–68		20	44 ± 10	24–64	
Serum CA242 level								
> 20 U/ml	29	25 ± 5	15–35	0.111	19	24 ± 6	12–36	0.018
⩽20 U/ml	40	49 ± 11	27–71		28	44 ± 12	20–68	
Differentiation								
G1	23	68 ± 9	50–86	<0.001	22	68 ± 26	17–119	<0.001
G2	28	40 ± 4	33–47		18	33 ± 6	20–46	
G3	18	14 ± 2	9–19		7	13 ± 7	0–26	
Staining score of LAPTM4B-35								
0	16	68 ± 9	50–86	<0.001	14	68 ± 23	23–113	<0.001
1	8	50 ± 9	34–67		7	46 ± 8	30–62	
2	26	26 ± 3	20–32		22	20 ± 1	18–22	
3	19	13 ± 1	10–16		4	14 ± 5	5–23	
Distant metastasis								
Present	14	10 ± 1	8–12	<0.001				
Absent	55	42 ± 6	29–55					
Resection type								
Radical (R0)	47	42 ± 6	30–54	<0.001				
Palliative (R1)	22	11 ± 2	8–14					

GBC, gallbladder carcinoma; OS, overall survival; DFS, disease-free survival; SE, standard error; CI, confidence interval; AC, adenocarcinoma; ASCC, adenosquamous cell carcinoma; SCC, squamous cell carcinoma; CEA, carcinoembryonic antigen; CA, carbohydrate antigen; LAPTM4B, lysosome-associated protein transmembrane-4 beta; G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated.
a Log-rank test. The unit for the survival time was 'month'.

Table 3 – Independent factors affecting overall and disease-free survival of patients with GBC

Variables	OS			DFS		
	RR	95%CI	P^a	RR	95%CI	P^a
Histology type	4.743	1.692–13.295	0.003			
Tumour size	4.530	1.161–17.665	0.030	6.020	1.133–31.993	0.035
LNI				4.330	1.372–13.668	0.012
Distant metastasis	30.965	4.898–195.768	<0.001			
Nevin staging	3.717	1.824–7.575	<0.001	4.907	2.147–11.216	<0.001
Score of LAPTM4B-35	2.853	1.391–5.850	0.004	2.443	1.104–5.405	0.027

GBC, gallbladder carcinoma; OS, overall survival; DFS, disease-free survival; RR, relative risk; CI, confidence interval; LNI, lymph node involvement; LAPTM4B, lysosome-associated protein transmembrane-4 beta.
a Cox regression test.

ical and pathological characteristics such as tumour size and Nevin staging ($P < 0.05$; Table 3).

4. Discussion

Gallbladder carcinoma (GBC) was generally recognised as a life-threatening malignancy because of its dismal prognosis,[20] although it was obviously improved through radical resection according to some authors.[21] Therefore, many molecules involved in gallbladder carcinogenesis have been identified in published papers[7–12] and more clues are still expected. To our best knowledge, this is the first report referring expression and significance of *LAPTM4B*, a gene first cloned in HCC, in GBC. We showed by immunohistochemistry that LAPTM4B-35, a protein encoded by the gene, highly expressed in a majority of patients (76%) with GBC. It was demonstrated that *LAPTM4B* encoded two proteins, LAPTM4B-35 and LAPTM4B-24, because its ORF contained two ATGs. LAPTM4B-35 was translated from the first ATG at nt 157, whereas LAPTM4B-24 from the second one at nt 430.[14] Interestingly, the expression and roles of these two proteins in HCC were quite different. Following the phenomenon that the expression of LAPTM4B under the mRNA level was much higher in the patients with poorly differentiated HCC, in contrast to those with well or moderately differentiated ones,[14] the expression of LAPTM4B-35, rather than LAPTM4B-24, was further suggested to be closely associated with differentiation grade of HCC.[15,16] In the present study, the expression of LAPTM4B-35 significantly related to some clinical and pathological characteristics, such as histology type, lymph node involvement, distant metastasis, staging and differentiation of GBC. The first discovered phenomenon is the higher proportion of LAPTM4B-35 expression in adenocarcinoma, compared with other histological types. Mechanisms about this remain to be clarified. These data verified the previous results in HCC and suggested the relationship between highly expressed LAPTM4B-35 protein and unfavourable biological behaviors of GBC. Thus, it could be inferred that the *LAPTM4B* gene might play a pivotal role in progression of some solid malignancies, such as HCC and GBC. Currently, there have been some clues that are able to help to explain its mechanisms. It was shown that transfection of the *LAPTM4B* gene promoted anchorage independent growth of HLE cells,[14] whereas antisense oligonucleotides against *LAPTM4B* inhibited proliferation of BEL-7402, a HCC cell line in which *LAPTM4B* expression was observed.[13] As described by some authors,[16] overexpression of LAPTM4B-35 could activate some proto-oncogenes, such as c-myc, c-jun and c-fos, and promote malignant transformation in some cell lines. So the *LAPTM4B* gene might function as a proto-oncogene via its whole ORF translating product, LAPTM4B-35 protein. Moreover, a high homology (46%) indicated that *LAPTM4B* might have a similar multidrug-resistant phenotype identified in LAPTM4A.[22] Therefore, targeted inhibition of *LAPTM4B* at gene and/or protein levels might be a new idea for gene therapy of GBC. Certainly, further strong supports from basic investigations are needed.

Recently, some significant prognostic factors of GBC, including clinicopathological characteristics and some molecular markers, were identified in published papers.[10,11,23–25] Among them, newly introduced molecular markers might provide basis for further study and practical value. What calls for special attention is that these molecules, such as cyclin D1 and $p27^{Kip1}$,[10,11] are factors that are also involved in pathways of cell proliferation. Discoveries from the current study offer a novel candidate, *LAPTM4B*, which is also intensively associated with cell proliferation based on abovementioned data from HCC. Uni- and multivariate analysis revealed that patients with high LAPTM4B-35 staining scores survived for a much shorter time than those with low ones, and LAPTM4B-35 staining score served as an independent predictor of both overall and disease-free survival of GBC, along with conventional clinicopathological parameters. These results suggested that overexpression of LAPTM4B-35 was of prognostic significance in GBC. Therefore, it might be a helpful variable in predicting surgical outcome of GBC. In the future, further efforts should be made as to whether combined detection of LAPTM4B-35 and other molecules, such as cyclin D1, would be more valuable in enhancing prediction efficiency.

In conclusion, LAPTM4B-35 is overexpressed in a great proportion of patients with GBC. Its overexpression closely correlates with clinicopathological features and post-resectional survival of GBC. *LAPTM4B* gene might thus play a crucial role in progression and invasion and be of significant potential in being a novel target candidate for gene/protein inhibition in GBC.

Conflict of interest statement

None declared.

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