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Expression of lysosome-associated protein transmembrane 4B-35 in cancer and its correlation with the differentiation status of hepatocellular carcinoma

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Abstract

AIM: To produce high-quality polyclonal antibody to lysosome-associated protein transmembrane 4B-35 and to identify LAPTM4B-35 expression in cancer tissues and its correlation with differentiation status of hepatocellular carcinoma (HCC).

METHODS: The 297 bp 5′ end of *LAPTM4B* cDNA was obtained by PCR and inserted into prokaryotic expression vector pGEX-KG. Then the recombinant pGEX-KG-N_{1-99} was transformed into *E.coli* JM109 to express GST-fusion protein. The fusion protein was purified by glutathione sepharose™ 4B agarose. The purified GST-LAPTM4B-N_{1-99} was characterized by SDS-PAGE, and used to immunize rabbits. The titer and specificity of antisera were detected by ELISA and Western blot, respectively. The correlation between the expression levels of LAPTM4B-35 and the differentiation status of HCC was analyzed via Western blot. The expression of LAPTM4B-35 in HCC and other six cancer tissues was investigated via tissue chip and immunohistochemical analysis.

RESULTS: About 6.2 mg of pure GST-LAPTM4B-N_{1-99} was isolated from 1 L of bacteria. The GST-LAPTM4B-N_{1-99} produced high titer antisera in rabbits and showed good immunity. Western blot showed specific reactions for the antibody to the LAPTM4B-35 in the total proteins from HCC tissues and BEL-7402 cells, also to the fusion protein purified or in the transformed bacteria. LAPTM4B-35 was remarkably expressed in several cancers, such as HCC, breast cancer, gastric carcinoma, lung cancer, and colon carcinoma, but not commonly expressed in esophageal cancer and rectum carcinoma. Notably, the expression levels of LAPTM4B-35 were significantly and inversely correlated to the differentiation of HCCs in a 20 case analysis.

CONCLUSION: Specific polyclonal antibody (LAPTM4B-N_{1-99}-pAb) to LAPTM4B-35 was produced. It identified the expression of LAPTM4B-35 in some cancer tissues originated from single layer cuboidal and columnar epithelial cells and firmly demonstrated that the expression of LAPTM4B-35 in HCC was inversely correlated with the differentiation of HCC.

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Key words: LAPTM4B; LAPTM4B-N_{1-99}-pAb; HCC; Tissue microarray

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INTRODUCTION

Hepatocellular carcinoma (HCC) remains one of the commonest internal malignancies of mankind. It ranks fifth in frequency in the world with an estimated number of 0.5-1 million cases per year, most of which occur in sub-Saharan Africa and south-east Asia including China[1-3]. The vast majority can be attributed to chronic hepatitis B virus (HBV) infection and there has been almost no change in incidence. It has also become evident that the number of HCC is increasing in low to middle-incidence countries such as USA[4-6], France[7,8], UK[9,10] and Japan[11,12]. The rising number of cases in these countries is most likely to be due to chronic hepatitis C virus (HCV) infection[4,6,12].

LAPTM4B, which was first cloned by our research group as a novel oncogene candidate, is highly expressed in vast majority of HCCs[13,14]. BLAST program analysis showed that the LAPTM4B was mapped to chromosome 8q22.1, the gain region in HCC shown by CGH analysis. The ORF of LAPTM4B contains two ATGs, indicating that this gene may encode two putative proteins with 35 kDa (317 aa) and 24 kDa (226 aa)[14,15], respectively. The former study confirmed that the LAPTM4B-35 was translated from the whole ORF and initiated from the first ATG, whereas the LAPTM4B-24 was translated from the second ATG[14]. The integral membrane protein characteristics were indicated by computer

analysis and demonstrated by experiments. The *LAPTM4B* gene was widely expressed in normal human tissues shown by Northern blot[14]. Its expression was high in heart, skeletal muscle and testis; moderate in ovary, kidney; and pancreas; low in liver, spleen, and thymus; but lowest in lung and peripheral leukocytes. It was remarkably overexpressed (48 over 55 cases) in HCC when compared with PNL. Furthermore, the expression levels of *LAPTM4B* mRNA were significantly related to the differentiation status of HCCs: the highest in poorly-differentiated HCCs, higher in moderately-differentiated HCCs, and low in well-differentiated HCCs[14].

The biological effects of LAPTM4B were studied by transient and stable transfection. The result showed that cell proliferation was promoted via LAPTM4B stable transfection of both mouse NIH3T3 cells[16] and human HLE cells (manuscripts in preparation). Also, the LAPTM4B transfected NIH3T3 cells were tumorigenic when the transfectants were inoculated into NIH mice. Coimmunoprecipitation assay indicated that LAPTM4B interacted with integrin-$\alpha 6\beta 1$ in BEL-7402 cells, which were enhanced by LN-1[15], and might play an important role in the integrin-$\alpha 6$ mediating signal transduction pathways. It was also found that the sequences of 91 amino acids at the N-terminus of LAPTM4B-35 were essential for its functions on cell survival and growth, which was revealed via transient transfection of plasmids containing full length and truncated sequences (273 bp) at the 5' end of LAPTM4B ORF into HLE cells[14]. After 2-3 wk of G418 selection, colonies in pCDNA3-BE (containing truncated ORF) transfected cells were almost completely disappeared, whereas the pCDNA3-AE (containing full ORF) transfected cells formed lots of colonies[14]. These results indicate that LAPTM4B-35 plays an important role in the regulation of cell survival, proliferation, and may involve in carcinogenesis.

It was evidenced that the overexpression of LAPTM4B-35 promoted malignant transformation of some cell lines, including accelerated proliferation, migration and invasion of cells, and activated some protooncogenes, including immediate early genes, such as c-myc, c-fos and c-jun (manuscripts preparation).

To investigate the function and expression of LAPTM4B-35 in HCC and HCC cell lines, specific antibody to LAPTM4B-35, but not LAPTM4B-24, the 297 bp at 5'end of LAPTM4B cDNA encoding LAPTM4B-N_{1-99} was cloned into donor vector pGEX-KG[17-19] and the recombinant plasmid was transformed into competent *E. coli* cells JM109. The GST-LAPTM4B-N_{1-99} fusion protein was produced in JM109 cells after induced with IPTG, and purified using glutathione sepharose™ 4B agarose[20,21]. After rabbits were immunized, specific polyclonal antibodies, LAPTM4B-N_{1-99}-pAb, against the N-terminus of LAPTM4B-35 were obtained. With this antibody, the expressions of LAPTM4B-35 in HCC and several cancer tissues were performed via Western blot and TMA[21-24], respectively. The correlation between expression levels of LAPTM4B-35 and HCC differentiation status were analyzed.

MATERIALS AND METHODS

Subjects

A total of 20 pairs of specimens were obtained from patients with HCC (aged from 35 to 70 years) who underwent hepatectomy at the Peking Union Medical College Hospital (the West Hospital) in Beijing. Histopathological analyses were independently performed by pathologists. Tumor differentiation was graded as I-III, according to the Edmondson Grading System[25,26]. Specimens were frozen immediately after surgical resection and stored in liquid nitrogen.

Chemicals

Molecular biological enzymes were purchased from Sangon Biotechnology Ltd. Peroxidase-conjugated goat anti-rabbit IgG (H+L) was purchased from Zhongshan Biotechnology Ltd. Glutathione-sepharose 4B agarose was purchased from Pharmacia Biotech Ltd.

Tissue microarray (TMA)

A TMA slide was purchased from Chengdu Phargentech Ltd. It contained 60 species of tissues, including two normal esophageal epithelial and eight esophageal cancer tissues, two normal mammary gland and eight breast cancer tissues, two normal lung and eight lung cancer tissues, two normal gastric epithelial and eight gastric carcinoma tissues, two normal colonic epithelial and eight colon carcinoma tissues, two normal rectal epithelial and eight rectum carcinoma tissues. All specimens were embedded in paraffin.

Cloning of LAPTM4B cDNA encoding LAPTM4B-N_{1-98}

To clone the 5' end of LAPTM4B ORF, PCR method was used. Based on the published *LAPTM4B* gene sequence[14], two oligonucleotide primers (P1: 5' GGGATCCGCCACCATGACGTCACGGACTCGG 3'; P2: 5' GCGAAGCTTCGTCCAGGGCGCGACCATC 3') were synthesized (Sangon Biotechnology Ltd). P1 was extended to the 5'end containing recognition sequences for endonuclease *Bam*HI (underlined). P2 was extended to the 5'end containing recognition sequences for endonuclease *Hind* III (underlined). PCR amplification was performed for 30 cycles at 94 ℃ for 30 s, at 55 ℃ for 30 s and at 72 ℃ for 50 s.

Expression vector construction

The pGEX-KG vector was digested with *Bam*HI and *Hind* III. cDNA fragment at the 5' end of *LAPTM4B* ORF was subcloned into the corresponding site of pGEX-KG to obtain a plasmid pGEX-KG-N_{1-99}. Then the recombinant plasmid was transformed into the *E.coli* strain JM109. The transformed colonies containing the cloned cDNA were screened by restriction enzyme analysis and DNA sequencing.

Expression and purification of GST-LAPTM4B-N_{1-99} in E.coli

An overnight LB culture of *E.coli* containing pGEX-KG-N_{1-99} was inoculated at a dilution of 1:100, and incubated at 37 ℃ with shaking. When the absorbance at 600 nm reached 0.6, GST-LAPTM4B-N_{1-99} expression was induced by the addition of 0.5 mmol/L IPTG. Further 4 h for growing, the *E.coli* strains were harvested by spinning at 4 000 r/min for 15 min at 4 ℃. The pellet was resuspended in PBS containing 0.05% Tween-20 of the original volume and lysed by sonication on ice, and then centrifuged at 10 000 *g* for 20 min at 4 ℃. The supernatant was passed through glutathione-sepharose 4 B beads according to the manufacturer's instructions. SDS-PAGE was performed to analyze the purified protein from the infected cells.

Immunogenicity assay on recombinant protein

Two rabbits were used to generate polyclonal antibodies against the fusion protein. Each rabbit was injected with 1 mg purified fusion protein in complete adjuvant in the initial injection and with uncompleted adjuvant for the following injection. Before injection, the rabbit blood was collected for producing pre-immune serum. The titer of antisera was measured by ELISA.

Western blot

Proteins in cell lysates and tissues were fractionated by 10% SDS-PAGE and followed by electrotransferring onto nitrocellulose filters (Bio-Rad). The filters were blocked at 4 ℃ overnight with a blocking buffer (pH 7.6) containing 5% non-fat dry milk. Then the filters were incubated with indicated antibody (1∶300) for 2 h at room temperature. After being washed with TBST (pH 6.0), the filters were incubated at room temperature for 1 h with horseradish peroxidase conjugated goat anti-rabbit IgG at 1∶2 000 dilution in TBS-0.5% non-fat dry milk. Immunoreactive bands were visualized using ECL detection reagents (Santa Cruz).

Immunohistochemistry

HCC tissue and TMA slide were fixed in 4% PFA in PBS for 10 min, washed twice with PBS, and incubated in 3% H_2O_2 to eliminate the endogenous peroxidase activities. The tissues were then incubated either at room temperature for 1 h or at 4 ℃ overnight with 1% goat serum in PBS to block the nonspecific binding of antibodies. The slides were further incubated sequentially with polyclonal antibody and goat anti-mouse IgG conjugated to horseradish peroxidase. The pre-immune serum was used as a control. Color developments were performed by incubation with 3,3'-diaminobenzidine tetrahydrochloride in 0.03% H_2O_2 and 50 mmol/L Tris-HCl, pH 7.4. Hematoxylin was used for counterstaining.

RESULTS

Expression of fusion protein and protein purification

Confirmed by restriction endonuclease digestion and DNA sequencing, pGEX-KG-N_{1-99} plasmid was successfully constructed and GST-LAPTM4B-N_{1-99} was expressed in *E.coli* JM109 by IPTG induction. A 36 kDa fusion protein was stained in high intensity by 10% SDS-PAGE (Figure 1). This fusion protein was majored as a soluble pattern.

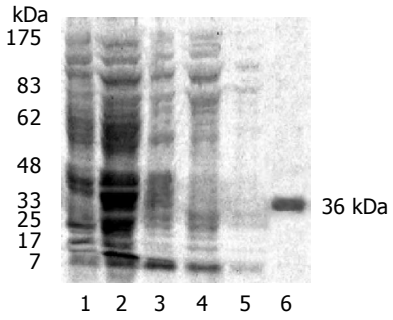

Figure 1 Purification of GST-LAPTM4B-N_{1-99}. 1: Uninduced *pGEX-KG-N_{1-99}*/JM109; 2: induced *pGEX-KG-N_{1-99}*/JM109; 3: supernatant of induced *pGEX-KG-N_{1-99}*/JM109; 4: unbinding fragement of glutathione sepharose™ 4B column; 5: eluated by PBS; 6: purified fusion protein GST-LAPTM4B-N_{1-99}.

Titer and specification of LAPTM4B-N_{1-99}-pAb

After being immunized thrice with purified GST-LAPTM4B-N_{1-99}, two rabbits both generated antisera at a titer of 5×10^4, measured by ELISA. Western blot analysis showed that there was a specific Ag-Ab binding band at 36 kDa in the unpurified and purified GST-LAPTM4B-N_{1-99} (Figure 2A, lanes 1 and 2). The BEL-7402 cell lysate was also identified by LAPTM4B-N_{1-99}-pAb (Figure 2B, lane 4).

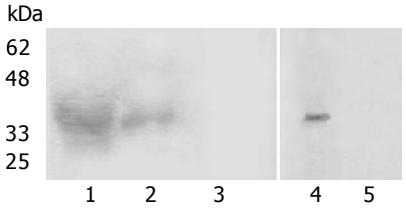

Figure 2 Western blot analysis of the specificity of LAPTM4B-N_{1-99}-pAb. 1: pGEX-KG-N_{1-99}/JM109 induced with IPTG (36 ku); 2: Purified infused protein (36 ku); 3: pGEX-KG-N_{1-99}/JM109 induced without IPTG; 4: BEL-7402 cell lysis, Western blot by LAPTM4B-N_{1-99}-pAb; 5: BEL-7402 cell lysis, Western blot by preimmunized antisera.

Expression analysis of LAPTM4B-35 in HCC

The expression levels of LAPTM4B-35 were significantly related to the differentiation status of HCCs: highest in poorly-differentiated HCCs (grade III, 12/20), high in moderately-differentiated HCCs (grade II, 4/20), and low in well-differentiated HCCs (grade I, 4/20) (Figure 3).

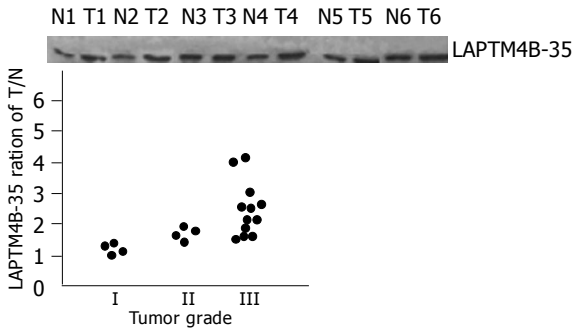

Figure 3 Analysis of the expression of LAPTM4B-35 with the differentiation status of HCCs. (a) Western blot analysis of LAPTM4B-35 in HCC tumor tissues (T), paired noncancerous liver tissues (N). (Tumor grade I: T3,T5; grade II: T1, T6; grade III: T2,T4) (b) Correlation between LAPTM4B-35 protein level and tumor grade. Paired tumor (T) *vs* adjacent noncancerous liver tissues (N) from 20 HCC patients were compared for their LAPTM4B expression by Western blot. Each spot in the figure represents the ration (T/N) of the LAPTM4B-35 expression (tumor *vs* adjacent noncancerous liver tissue) from one patient (P<0.05).

Immunohistochemical analysis of LAPTM4B-35 on HCC tissue and TMA

Immunohistochemical analysis was undertaken to characterize the expression of LAPTM4B-35 in HCC and TMA. In HCC (Figure 4A), positive brown signals were seen only in HCC cells and negative signals in the interstitial tissue of liver. The result was well coincident with the mRNA detection via in situ hybridization (Figure 4C)[14]. Moreover, the expression of LAPTM4B-35 was also analyzed via TMA in six types of cancer tissues: esophageal carcinoma, breast cancer, pulmonary carcinoma, gastric carcinoma, colon carcinoma,

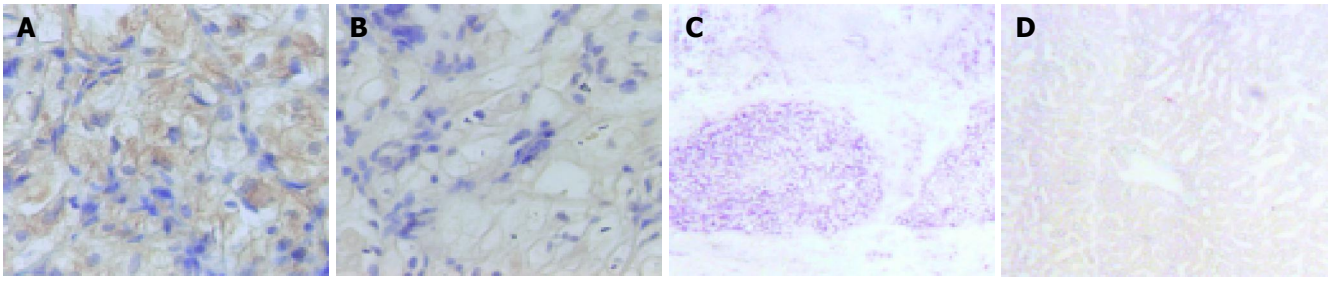

Figure 4 Analysis of LAPTM4B-35 and LAPTM4B mRNA expression by ISH in HCCs. **A:** immunized antisera (20×); **B:** preimmunized antisera (20×); **C:** LAPTM4B mRNA expression in HCCs (20×); **D:** paired noncancerous tissue (20×).

and rectum carcinoma. There were no significant LAPTM4B-35 expressions in six counterparts of normal tissues but obvious high and weak expression accounted for the majority of breast cancers and gastric carcinomas. Positive staining could only be observed in minority of pulmonary carcinomas and colon carcinomas and almost negative staining in all of esophageal carcinomas and rectum carcinomas tested (Figure 5).

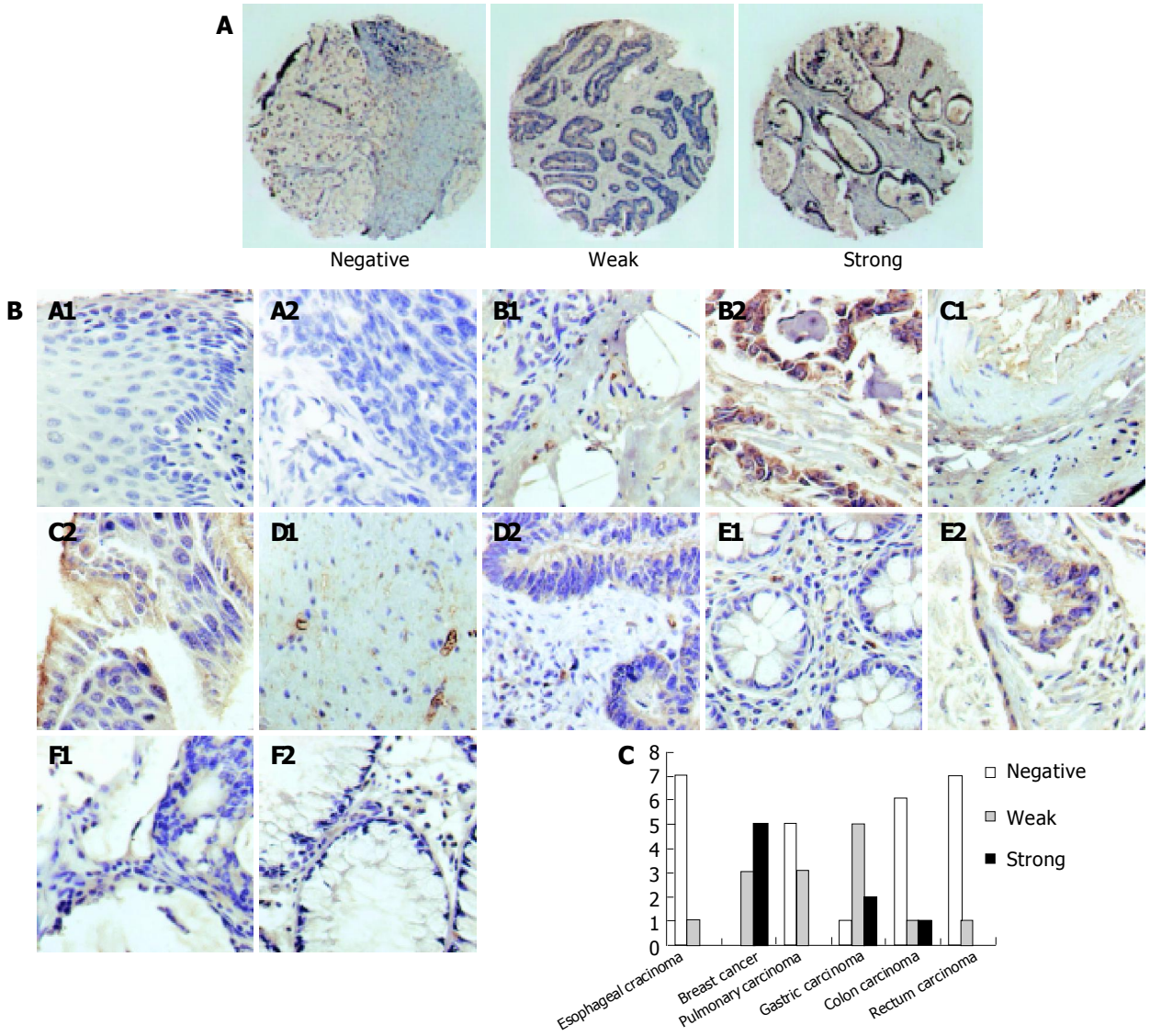

Figure 5 Analysis of LAPTM4B-35 via TMA. **A:** Scores assigned to each tissue spot according to three different staining coverages. Negative represents the complete negative staining. Weak represents scores assigned to tissue disks with borderline and partial positive staining. The complete positive staining was designated as strong; **B:** Representative elements of a TMA stained with LAPTM4B-$N_{1\text{-}99}$-pAb. Magnification ×200. Normal tissue stained with LAPTM4B-$N_{1\text{-}99}$-pAb (A1: esophageal mucous; B1: mammary gland tissue; C1: normal lung tissue; D1: gastric mucous; E1: colonic mucous; F1: rectal mucous). Tumor tissue stained with LAPTM4B- $N_{1\text{-}99}$-pAb (A2: esophageal carcinoma; B2: breast cancer; C2: pulmonary carcinoma; D2: gastric carcinoma; E2: colon carcinoma; F2: rectal carcinoma.); **C:** Histogram of LAPTM4B expression assessed using 6 TMAs.

DISCUSSION

In our previous work, we have produced a polyclonal antibody: LAPTM4B-EC2-pAb, which was prepared by immunization with KLH-conjugated 10-peptides whose sequences are localized at the second extracellular loop between the third and fourth transmembrane regions. By Western blot, expressions of LAPTM4B-35 and LAPTM4B-24 were identified in HCC, PNL and NL tissues with LAPTM4B-EC2-pAb, indicating that LAPTM4B-35 may initiate from the first ATG, whereas LAPTM4B-24 is translated from the second ATG. Notably, the expression levels of LAPTM4B-35 were primarily shown by Western blot with LAPTM4B-EC2-pAb to correlate with the differentiation status of 12 cases of HCC tissues, but the expression levels of LAPTM4B-24 did not relate to the differentiation status of HCC tissues.

To further clarify the correlation between upregulation of LAPTM4B-35 and differentiation of HCC and the function of LAPTM4B-35, we generated a specific antibody: LAPTM4B-N_{1-99}-pAb, against LAPTM4B-35 but not against LAPTM4B-24. Applying this antibody, it was firmly demonstrated that the expression of LAPTM4B-35 protein was inversely related with the status of differentiation status of HCC, suggesting its importance in carcinogenesis and progress of HCC as well as a possibility of being a criterion of HCC differentiation and pathological grading. The expression levels of LAPTM4B-35 were not found to correlate with the AFP levels of sera.

Using LAPTM4B-N_{1-99}-pAb, the expression of LAPTM4B-35 in some cancers originated from single layer cuboidal and columnar epithelia, such as HCC, breast cancer, gastric cancer and pulmonary cancer. We have previously reported that the high metastatic cell lines, such as the giant cell pulmonary carcinoma and prostate carcinoma cell lines highly expressed LAPTM4B-35. However, cancers originated from stratified epithelia, such as esophageal carcinoma and rectum carcinoma, did not practically express LAPTM4B-35. Malignant tumors from mesenchymal cells did not express LAPTM4B-35 either. The result of TMA gave us a clue that LAPTM4B-35 might also play an important role in breast cancer, gastric cancer or pulmonary carcinoma.

In conclusion, specific polyclonal antibody to the cytoplasmic N-terminal tail of LAPTM4B-35 was obtained and used for Western blot and immunohistochemistry. The expression of LAPTM4B-35 is associated with the differentiation status of HCC. In addition, the expression of LAPTM4B-35 in other cancer tissues may pave the way for further study on the function of N-terminal of LAPTM4B-35 for the development of tumors and their clinical diagnosis.

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