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LAPTM4B-35, a novel tetratransmembrane protein and its PPRP motif play critical roles in proliferation and metastatic potential of hepatocellular carcinoma cells

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Lysosomal protein transmembrane 4 beta (*LAPTM4B*) was originally identified as a hepatocellular carcinoma (HCC)-associated gene. This gene and its protein product LAPTM4B-35, are both overexpressed in a variety of human cancers. However, its specific role in cell transformation and malignancy has remained elusive. In the present study we investigated the effects of LAPTM4B-35 overexpression on the malignant phenotypic features in the HLE cell line. Our data show that overexpression of LAPTM4B-35 promotes cell proliferation, exogenous growth-stimulating factor-independent and anchorage-independent growth, and enhances metastatic potential, including promotion of both cell migration and invasion. Study of the underlying mechanisms demonstrated alterations of molecular events involved in these processes, which included upregulation of proliferation-promoting transcription factors such as c-Myc, c-Jun, and c-Fos, and cell cycle-promoting proteins such as cyclin D1 and cyclin E. In addition, mutagenesis study showed that the PPRP motif in the N-terminal region of LAPTM4B-35 plays a critical role in promoting proliferation, migration, and invasion, as well as in the upregulation of the oncoproteins noted above. These data offer insight into the mechanism by which this novel tetratransmembrane protein contributes to the pathogenesis of liver cancer, and suggest that it may be a potential target for cancer therapy. (*Cancer Sci* 2009; 100: 2335–2340)

Our previous studies reported a novel HCC-associated gene designated as *LAPTM4B* by HUGO Gene Nomenclature Committee. The full-length cDNA sequence (NCBI NM_018407, Gene ID = 55353) of *LAPTM4B* contains two translational initiation codons (ATG) with an interval of 273 bp as predicted by bioinformatics and, therefore, encodes two protein isoforms: LAPTM4B-35 and LAPTM4B-24 with apparent molecular weights of 35 and 24 kDa, respectively, which have been confirmed by western blot analysis.[1,2] The LAPTM4B proteins contain four transmembrane domains, two extracellular domains (EC1 and EC2), and two cytoplasmic tails consisting of the N-terminus and C-terminus of the protein, the latter of which contains typical lysosome-targeting motifs.

LAPTM4B-24 shows 46% homology to lysosomal protein transmembrane 4 alpha at the amino acid level.[1,3,4] Human LAPTM4B shares high homology (92%) with its murine counterpart. It also has certain homology to fish and other vetebrates, as well as to some invertebrates such as *Drosophila*. Therefore, LAPTM4B is a conserved protein in evolution.[1]

LAPTM4B mRNA is expressed in a wide range of human normal tissues to various extents; it is especially high in the testis, heart, skeletal muscle, and uterus, but very low in the liver and lungs. Notably, the level of LAPTM4B mRNA determined by northern blot analysis is markedly upregulated in 87.3% (48/55) of HCC, and correlates significantly with the pathological grade and differentiation of HCC.[1] LAPTM4B mRNA is also elevated in a variety of other carcinomas including lung cancer (88%, 23/26), colon cancer (67%, 18/27), uterus cancer (68%, 30/44), breast cancer (51%, 27/53), and ovarian cancer (69%, 11/16), and significantly overexpressed in adrenocorticotrophin-secreting adenomas and non-functioning pituitary adenomas by either northern blot or expression array analysis using Affymetrix GeneChip HG-U133A arrays (Affimetrix, Santa Clara, CA, USA).[5,6]

As shown by immunohistochemistry with an antibody specifically against the N-terminus of this protein, LAPTM4B-35 protein is upregulated in a wide range of cancers including HCC, breast cancer, gastric cancer, lung cancer, colon cancer,[7] gallbladder cancer,[8] extra hepatic cholangiocarcinoma,[9] and ovarian carcinoma.[10] Most importantly, the LAPTM4B-35 staining index correlates positively with a higher rate of metastases to lymph nodes and distant organs, and reversely with both the overall and cancer-free survival post-resection of patients with gallbladder cancer,[8] extra hepatic cholangiocarcinoma,[9] ovarian cancer,[10] and HCC[11]. Thus, LAPTM4B-35 could be used as an independent prognostic marker for patients with HCC, gallbladder cancer, extra hepatic cholangiocarcinoma, and ovarian cancer. Similarly, the protein level of LAPTM4B-35, but not LAPTM4B-24, is significantly upregulated as shown by western blotting in HCC and HCC-derivative cell lines including HepG2, SMMC-7721, QGY-7701, BEL-7402, and HG116.[1] Moreover, the ratio of LAPTM4B-35 to LAPTM4B-24 was markedly elevated in HCC compared with that in paired noncancerous liver (PNL) and normal liver (NL), suggesting that this ratio is important and may be associated with the development of HCC.[2]

The aim of the present paper is to study the roles played by LAPTM4B-35 in malignant cellular phenotype and the related molecular involvement by transfecting full-length ORF cDNA into the human HLE cell line, which uniquely expresses a relatively low level of *LAPTM4B*.[1] Acceleration and deregulation of proliferation by LAPTM4B-35 overexpression was evidenced. Some proliferation-promoting transcription factors and the related cell cycle-promoting cyclins were found to be increased in LAPTM4B-overexpressing HLE-AE cells compared to the control HLE-MOCK cells. Meanwhile, enhancement of metastatic potency was indicated by cell migration and invasion assay in HLE-AE cells.

Our previous studies implicated that, in contrast to LAPTM4B-35, LAPTM4B-24 might function to inhibit cell

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survival or growth.[1] Structurally, LAPTM4B-35 differs from LAPTM4B-24 in that it contains an extra 91 amino acid residues at the N-terminus. Therefore, cell proliferation promoted by LAPTM4B-35 may be associated with the 91-amino acid sequence. Moreover, this cytoplasmic region of LAPTM4B-35 harbors a proline-rich motif, PPRP, which could be the binding site for a variety of signaling molecules that are involved in the activation of oncogenic signal pathways.[12] Based on the above information we investigated in this study the role played by the PPRP motif and evidenced by mutagenesis its importance in the regulation of cell growth, proliferation, and migration. Together, the overexpression of LAPTM4B-35 and its PPRP motif contribute to the advancement of malignant proliferation and metastatic potency of human HCC cells.

Materials and Methods

Cell lines and antibodies. The HLE cell line, which was originated from the HCC of a 68-year-old patient,[13] was a gift from Professor Hayashi Yoshitake, Kobe University (Japan), and kept in our laboratory. The L02 (HL-7702) cell line was purchased from the Shanghai Institute of Cell Biology of the Chinese Academy of Science and kept in our laboratory. Anti-LAPTM4B-N10 or anti-LAPTM4B-EC2 polyclonal antibodies against epitopes in the N-terminus or EC2 domain of LAPTM4B-35 were produced and purified sequentially by Protein A-Sepharose and antigen–peptide affinity chromatography in our laboratory. Anti-cyclin D1 mAb, anti-c-Myc mAb, anti-cyclin E, anti-c-Fos, and anti-c-Jun polyclonal antibodies were all from Santa Cruz, CA, USA.

Plasmid construction and transfection. The plasmids *pcDNA3/LAPTM4B-AE* and *pcDNA3/LAPTM4B-BE* containing full-length *LAPTM4B* ORF cDNA, or truncated cDNA initiated from the second ATG of *LAPTM4B* ORF, respectively, were constructed as previously described.[1] The PPRP mutatant plasmid *pcDNA3/LAPTM4B-PA* containing a replacement mutation *(P12,13,15A)* of *LAPTM4B-AE* was constructed by two-step PCR amplification. The primers used were: LAPTM4B-F, 5′-GGGATCCGCCACCATGACGTCACGGACTCGGGTC-3′; LAPTM4B-R, 5′-CGGAATTCGGCAGACACGTAAGGTGGC-GG-3′; PA-1, 5′-GAGGGCGCGGGCCGCACTCGGCCATG-TGACCCGAGT-3′; and PA-2, 5′-CACATGGCCGAGTGCGG-CCCGCGCCCTCCCCGTCC-3′.

Transfections were done with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Positive clones were selected with G418 (Gibco BRL) at a concentration of 700 μg/mL. The stable transfectants from *pcDNA3* empty plasmids, *pcDNA3/LAPTM4B-AE* plasmids, and *pcDNA3/LAPTM4B-PA* plasmids were designated herein as HLE-MOCK, HLE-AE, and HLE-PA, as well as L02-MOCK, L02-AE, and L02-PA, respectively. Each of the stable transfectants used in this study was a pool of mixed colonies obtained after G418 treatment for killing the cells that were not successfully transfected by the respective plasmids.

Protein extraction and western blotting. Protein extraction and western blotting were carried out as described previously.[1]

Cell growth curve assay. One thousand cells were seeded into 96-well plates and cultured in the presence of 10 or 1% bovine serum. For the cell growth curve assays of HLE-MOCK, HLE-AE, and HLE-PA cells (Fig. 1b), FBS was used. For the exogenous stimulating factor-independent growth assay (Fig. 2a) BBS was applied. The selection of variant sera was to maintain a suitable proliferation level of HLE-MOCK cells as each control. The viable cells were counted by APA[14] for 4 days with an interval of 24 h.

^{3}H-TdR incorporation. Cells were seeded into 96-well plates and incubated in DMEM with 10% FBS for 24 h, then medium containing ^{3}H-TdR (5 μCi/mL) was added into the plates for 24 h for incorporation. The rate of DNA synthesis (cpm value) was determined with a radio detection device.

Flow cytometry. Cells were harvested and fixed in 75% ethanol at 4°C overnight, then washed twice with PBS and treated with RNaseA (0.2 mg/mL) for 30 min at room temperature. Cells were then incubated with buffer containing 0.1% Triton X-100 and 0.02 mg/mL propidium iodide. The cell cycle was analyzed using FACScan and CELLQuest software (Becton Dickinson, Franklin Lakes, NJ, USA).

Anchorage-independent growth assay. The 0.67% soft agar gel was added into each well of a six-well plate as base layer. The upper layer containing 1000 cells per well was prepared with 0.3% soft agar gel. Colonies larger than 60 μm in diameter were counted after 14 days of culture.

Migration and invasion assay. A Boyden Chamber assay was applied as described by Lang *et al.*[15] Matrigel was prepared in our laboratory from the Engelbreth–Holm–Swarm tumor according to the method of Kleinman *et al.*[16]

Statistical analysis. Statistical analysis was carried out using Student's *t*-test. A P-value <0.05 was considered statistically significant. All experiments were done in triplicate and repeated at least twice.

Results

Characterization of LAPTM4B transfectants. The HLE-AE cells transfected with *pcDNA3/LAPTM4B-AE* plasmids containing the full-length ORF cDNA of *LAPTM4B* survived and grew well during the G418 selection; but the HLE-BE cells transfected with *pcDNA3/LAPTM4B-BE* plasmids gradually died out within 2–3 weeks of G418 selection. This data was consistent with the results of the colony formation assay in BHK cells,[1] suggesting that LAPTM4B-24 and LAPTM4B-35 might play an antagonistic role in cell survival and growth. Western blotting showed that both HLE-AE and HLE-PA cells markedly expressed a protein with an apparent molecular weight of 35 kDa (Fig. 1a), while they expressed a protein of 24 kDa at a very faint level. The HLE-BE cells expressed high levels of LAPTM4B-24 (data not shown).

LAPTM4B-35 overexpression promotes cell proliferation. To examine the effect of *LAPTM4B* on cell proliferation, three approaches were applied. Cell proliferation was first measured by growth curves. As shown in Figure 1(b), the growth rate of HLE-AE cells in which LAPTM4B-35 was overexpressed was faster than that of HLE-MOCK cells, indicating that overexpression of LAPTM4B-35 significantly accelerated the cell growth.

DNA synthesis was then detected by ^{3}H-TdR incorporation. As shown in Figure 1(c), the cpm of ^{3}H-TdR incorporation in HLE-AE cells increased up to approximately 2-fold of that in HLE-MOCK cells after 24 h of incubation, indicating that the DNA synthesis in LAPTM4B-35-overexpressing cells increased dramatically.

The cell cycle was analyzed by flow cytometry. As shown in Figure 1(d), the percentage of cells in S phase increased to 38% in HLE-AE cells compared to 22% in HLE-MOCK cells, whereas the percentages in G_0/G_1 and G_2/M phases of the HLE-AE cells were significantly reduced compared with those of HLE-MOCK cells. This result indicated that cell cycle progression, especially the G_1/S transition, was promoted in LAPTM4B-35-overexpressing cells.

Over all, it is obvious that cell proliferation was accelerated by LAPTM4B-35 overexpression.

LAPTM4B-35 promotes growth-stimulating factor-independent and anchorage-independent proliferation. Growth-stimulating factor-independent and anchorage-independent growth are two of the basic characteristics of malignant cells. The growth curves of HLE-AE and HLE-MOCK cells were compared in a

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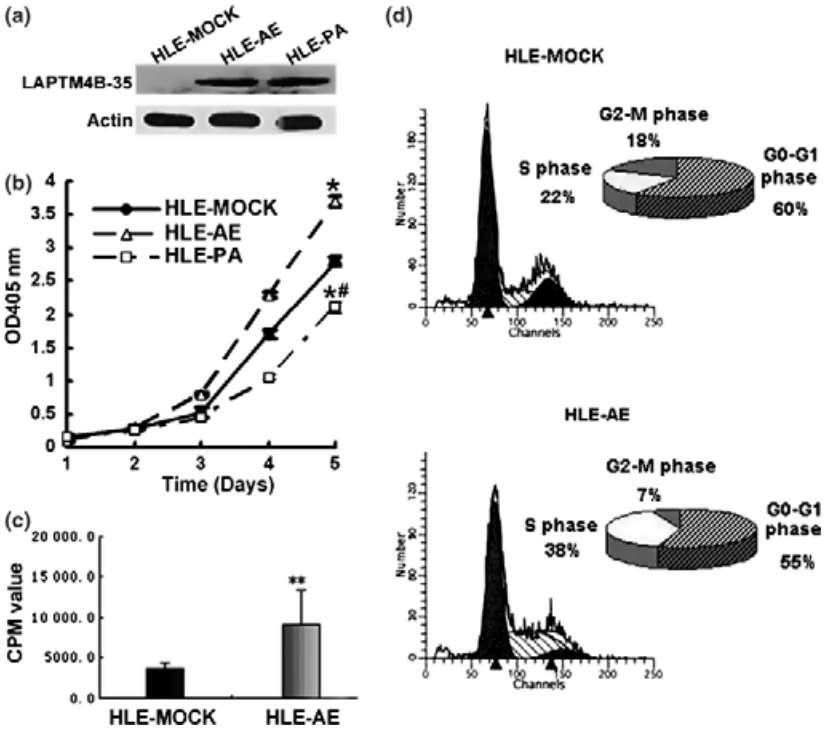

Fig. 1. Alterations of cell proliferation by LAPTM4B-35 overexpression or mutation (*P12,13,15A*) of LAPTM4B-35. (a) Identification of LAPTM4B-35 expression by western blotting with the anti-LAPTM4B-N10 polyclonal antibody in HLE-AE and HLE-PA cells, which were transfected with *pcDNA3/LAPTM4B-AE* plasmid containing the full-length ORF cDNA of *LAPTM4B* and *pcDNA3/LAPTM4B-PA* plasmid containing a replacement mutation *(P12,13,15A)* of *LAPTM4B-AE*, respectively. (b) Growth curves of HLE-AE and HLE-PA cells. Cells (1×10^3) were plated into 96-well culture plates and cultured in DMEM medium supplemented with 10% FBS. The viable cells were quantified by acid phosphatase assay[14] and optical density measured at 405 nm every 24 h during a 5-day period. Each point represents the mean ± SD from triplicate wells. The experiment was repeated three times. $*P < 0.05$ (HLE-AE and HLE-PA *vs* HLE-MOCK); $\#P < 0.01$ (HLE-AE *vs* HLE-PA). (c) Promotion of DNA synthesis by LAPTM4B-35 overexpression. DNA synthesis was determined by ^{3}H-TdR incorporation. Cells (2×10^4) were plated into each well of 96-well culture plates and incubated in DMEM medium supplemented with 10% FBS for 24 h, then medium containing ^{3}H-TdR (5 μCi/mL) was added into the wells for 24 h of incorporation. DNA synthesis (cpm value) was measured. The cpm counts represent the mean ± SD from triplicates and the experiment was repeated three times. $*P < 0.01$ (HLE-AE *vs* HLE-MOCK). (d) Promotion of the cell cycle by LAPTM4B-35 overexpression. The cell cycle was analyzed by flow cytometry. Cells were harvested and fixed in 75% ethanol at 4°C overnight, then washed twice with PBS and treated with RNaseA (0.2 mg/mL) for 30 min at room temperature. The cells were then incubated with buffer contain 0.1% Triton X-100 and 0.02 mg/mL propidium iodide. The cell percentage in each cell cycle phase was analyzed. The cell percentage in S phase was 38% of HLE-AE cells, compared with 22% of HLE-MOCK cells; that in G_0/G_1 phase was 55% of HLE-AE cells, compared with 60% of HLE-MOCK cells, indicating the promotion of cell cycle passage, in particular the transition of G1/S, by LAPTM4B-35 overexpression. The results were reproducible in multiple repeated experiments.

medium containing only 1% BBS, which is deficient in growth-stimulating factors for proliferation of cells with relatively lower malignancy but contains enough adhesion factors for cell attachment onto the surface of substrate, which is necessary for avoiding the ''anoikis'' caused by detachment of cells from the substrate surface. As shown in Figure 2(a), under 1% BBS-supplemented medium HLE-MOCK cells stopped growing, while HLE-AE cells sustained continuing growth and proliferation. The number of viable cells in HLE-AE was twice that of HLE-MOCK after 4 days of culture, indicating that overexpression of LAPTM4B-35 promotes exogenous growth-stimulating factor-independent proliferation.

Anchorage-independent growth *in vitro* is another phenotype of cell transformation and malignancy, and is tightly associated with tumorigenesis *in vivo*. To assess the effect of LAPTM4B-35 on anchorage-independent growth, colony formation in soft agar was carried out. As shown in Figure 2(b), the colony formation capacity of HLE-AE cells in soft agar was significantly enhanced compared with HLE-MOCK cells. After 7 days of culture, large-sized colonies were formed in HLE-AE cells, but no visible colony was found in HLE-MOCK cells. After 9 days, the HLE-AE colonies were significantly large in size and greater in number than those of HLE-MOCK cells (Fig. 2b). After 40 days of culture, most HLE-MOCK cells had died, whereas HLE-AE cells were still vigorously growing in the top-layer agar, and most colonies even grew out of the top-layer agar, forming a mountain-like appearance. These *in vitro* studies in tridimensional matrices showed that LAPTM4B-35 promotes progression of HLE cells toward advanced malignancy by further deregulating cell proliferation.

Upregulation of c-Myc, c-Jun, and c-Fos in LAPTM4B-35-overexpressing HLE-AE cells. c-Myc, c-Jun, and c-Fos are transcription factors that control cell proliferation, and are frequently aberrantly expressed in cancer cells. To test whether these transcription factors are affected by overexpression of LAPTM4B-35, we examined the protein levels by western blotting. Our data showed that c-Myc, c-Jun, and c-Fos were significantly enhanced in HLE-AE cells compared to HLE-MOCK cells (Fig. 2c, left), suggesting that these proliferation-promoting transcription factors, which are encoded by the early-response proto-oncogenes, may be involved in the deregulative proliferation of HLE-AE cells.

Upregulation of cyclin D1 and cyclin E in LAPTM4B-35-overexpressing HLE cells. Cyclin D1 and cyclin E are important cell cycle regulators that control G1 phase and the G/S transition of the cell cycle, respectively. To examine whether cyclin D and cyclin E levels are affected in LAPTM4B-35-overexpressing HLE-AE cells, western blotting was carried out. The results

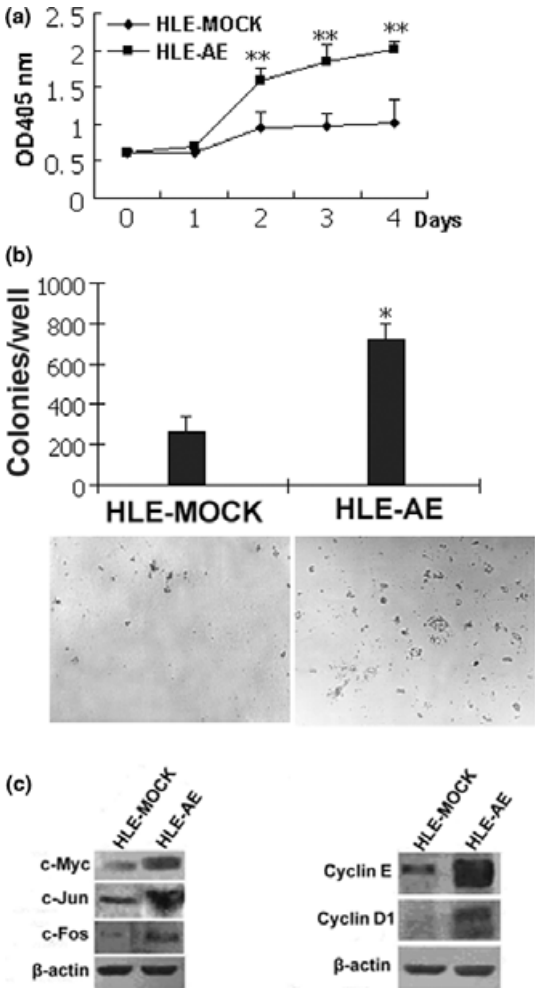

Fig. 2. Promotion of serum-independent and anchorage-independent proliferation by LAPTM4B-35 overexpression and the variation of molecules involved. (a) Promotion of serum-independent proliferation by LAPTM4B-35 overexpression. The viable cell number was quantified by acid phosphatase assay.[14] The HLE-AE cells continued to proliferate, whereas HLE-MOCK cells stopped growing under the same medium containing only 1% baby bovine serum (BBS), necessary for cell attachment onto the substrate surface to avoid ''anoikis''. The number of viable HLE-AE cells was twice that of HLE-MOCK after 4 days of culture. (b) Promotion of anchorage-independent proliferation by LAPTM4B-35 overexpression. HLE-AE and HLE-MOCK cells were cultured in 0.3% agar containing DMEM medium. Colonies were counted in six random fields per well of six-well culture plates. Upper panel: Histogram analysis of colonies with diameters greater than 60 μm after 9 days of culture. The data are presented as mean ± SD from triplicates. $^{**}P < 0.01$ HLE-AE cells *versus* HLE-MOCK cells. Lower panel: colony formation of HLE-AE and HLE-Mock cells in soft agar. The number and size of HLE-AE colonies formed in soft agar were larger than those formed by HLE-Mock cells, indicating that the colony formation potency of HLE-AE cells in soft agar was significantly enhanced compared with HLE-MOCK cells. (c) Upregulation of c-Myc, c-Jun, c-Fos, cyclin D1, and cyclin E proteins in HLE-AE cells measured by western blotting, $^{*}P < 0.05$.

(Fig. 2c, right) showed that the protein levels of cyclin D1 and cyclin E were dramatically upregulated up to four- and seven-fold, respectively, when compared with HLE-MOCK cells, indicating that these cell cycle regulating proteins are involved in the deregulative proliferation of HLE-AE cells.

LAPTM4B-35 mutation inhibits cell proliferation and expression of oncoproteins. The promotion of proliferation by LAPTM4B-35 may be contributed by the extra 91-amino acid fragment in the N-terminal cytoplasmic region of LAPTM4B-35, which contains the PPRP proline-rich motif PPRP that could serve as the binding site for SH3 domain-containing proteins that are involved in many signaling pathways.[12] It therefore may play a role in the formation of the malignant phenotype in cancer cells. To investigate the role of the PPRP motif in the function of LAPTM4B-35, the PPRP motif was mutated to AARA (P12,13,15A) and introduced into HLE cells labeled as HLE-PA. Our results showed that cell proliferation was significantly inhibited in HLE-PA cells that stably expressed the mutant LAPTM4B-PA (Fig. 1b). Consistent with this, the levels of cell cycle-promoting proteins, c-Myc and cyclin D1, were much lower when compared with wild-type HLE-AE cells (Fig. 3b, left panel). Additionally, similar results were obtained using L02 cells, a cell line originated from human normal liver cells. Cell proliferation and cell cycle-promoting cyclin E expression were enhanced when wild-type LAPTM4B-35 was introduced, but markedly inhibited when PA-mutated LAPTM4B-35 was introduced (Fig. 3a,b, right panel). These results imply that the PPRP motif plays a critical role in LAPTM4B-35 function in cell transformation and malignancy.

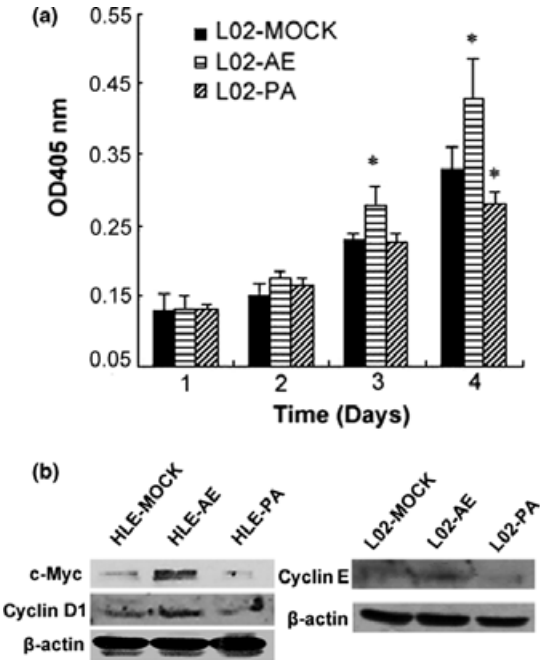

Fig. 3. Inhibition of cell proliferation and downregulation of some oncoproteins by mutation of the PPRP motif at the N-terminus of LAPTM4B-35. Cell proliferation was measured by acid phosphatase assay. The oncoproteins were determined by western blotting. Each experiment was repeated three times. (a) Acceleration of L02-AE cell proliferation and inhibition of L02-PA cell proliferation. $^{*}P < 0.05$ (L02-AE *vs* L02-MOCK and L02-PA *vs* L02-MOCK) (b) Left: downregulation of c-Myc and cyclin D1 in HLE-PA cells compared with upregulation in HLE-AE cells. Right: downregulation of cyclin E in L02-PA cells compared with upregulation in L02-AE cells.

LAPTM4B promotes cell migration and invasion. It is well known that active migration and invasion are important characteristics of malignant cells and are involved in the process of cancer metastasis. Directional migration and invasion of transfected HLE cells in response to chemoattractant were detected via the Boyden Chamber assay. As shown in Figure 4, more HLE-AE cells but less HLE-PA cells migrated across the filter and invaded through the Matrigel than HLE-MOCK cells ($P < 0.05$), indicating that the potency of both cell migration and invasion was enhanced when LAPTM4B-35 was overexpressed, but was strongly inhibited by PPRP mutation of LAPTM4B-35.

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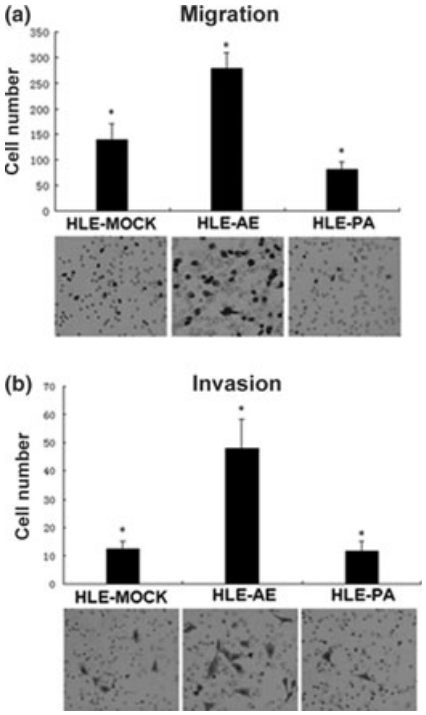

Fig. 4. Enhancement in HLE-AE cells and inhibition in HLE-PA cells of migration and invasion. Cell migration and invasion for HLE-AE and HLE-PA cells were detected by Boyden Chamber assay in the absence and presence of Matrigel, respectively. Total cells that migrated through the filter pores were counted and photographed after 18 h of culture for the migration assay and 20 h of culture for the invasion assay. (a) Cell migration profiles. (b) Cell invasion profiles. Upper panel: histogram analysis of cells that migrated through the filter pores. The data are presented as mean ± SD from triplicates. $*P < 0.05$. Lower panel: photographs of cells that migrated through the filter pores.

Discussion

Deregulation of proliferation and enhancement of migration/invasion are basic characteristics of cancer cells.[17] Although many oncogenes and oncoproteins have been found to be associated with HCC, the detailed mechanisms underlying HCC malignancy are not fully revealed. To search for unknown genes that are involved in the regulation of hepatocyte proliferation and tumorigenesis of HCC, fluorescence-labeled mRNA differential display was carried out using tissues from human normal liver, fetal liver, and HCC, as well as paired non-cancerous liver, each of them representing a different status of cell proliferation and/or differentiation. A novel HCC-associated gene, *LAPTM4B*, which is mapped to 8q22.1, was first cloned and identified in our laboratory. This gene and its encoded LAPTM4B-35 protein is markedly overexpressed in a vast majority of HCC tissues.[1,2]

In the present study the promotion of deregulated proliferation by LAPTM4B-35 overexpression was demonstrated by multiple approaches. All of the results showed that LAPTM4B-35 promotes cell proliferation in HLE-AE cells, as evidenced by accelerated growth rate, increased DNA synthesis, and accumulation of S-phase cells. Moreover, exogenous growth-stimulating factor-independent and anchorage-independent proliferation were also enhanced in HLE-AE cells, suggesting that LAPTM4B-35 overexpression plays an additional role in the progression of malignant phenotype in HLE-AE cells.

The regulation of cell proliferation is crucial in maintaining cellular homeostasis, and its loss is a principle hallmark of cancer cells.[17] An increased rate of hepatocyte proliferation in preneoplastic nodules is an early event in the progression of HCC. In the present study we explored the molecular mechanism underlying deregulative proliferation caused by overexpression of LAPTM4B-35. Our results showed that transcription factors such as c-Myc, c-Jun, and c-Fos were markedly increased in LAPTM4B-35-overexpressing cells. c-Myc is an oncogene associated with a variety of human cancers, and a high level of constitutive Myc protein can result in transcriptional activation of proliferation-promoting oncogenes and repression of growth arrest genes, and contribute to the progression and maintenance of a wide range of human cancers.[18–24] Given the importance of c-*myc* in the oncogenesis of human cancers,[23] our finding that c-myc is elevated in LAPTM4B-35-overexpressing HLE-AE cells has significant implications in understanding the role of LAPTM4B-35 in the tumorigensis of HCC. Consistent with this finding, c-Myc has been demonstrated to be overexpressed in most HCC and the *c-myc* gene has been shown to be amplified in 30% of HCC.[18,19] AP-1 (the heterodimeric transcription factor composed of c-Jun and c-Fos) is upregulated in a large number of human carcinomas, and activation of AP-1 is intricately involved in cell transformation; meanwhile, the requirement for c-Jun and c-Fos was restricted to early stages of tumorigenesis, and both the number and size of hepatic tumors were dramatically reduced when c-*jun* was inactivated after the tumor was initiated.[25–28] Therefore, it is possible that LAPTM4B-35 may participate in the process of malignant transformation and tumorigenesis through upregulation of c-Myc, c-Jun, and c-Fos.

As transcription factors, c-Myc, c-Jun, and c-Fos regulate the expression of genes essential for cell proliferation and differentiation. A number of studies suggest that c-Myc directly activates the expression of cyclin E and cyclin D1,[28,29] and that the AP-1 complex consisting of c-Jun and c-Fos regulates directly or indirectly the expression of some cell cycle-regulatory proteins, including cyclin E and cyclin D1.[27–31] The cyclin D1–CDK4/6 complex promotes the passage of G_1 phase of cell cycle and the cyclin E–CDK2 complex promotes the transition from G_1 to S phase.[31] Our results showed that both cyclin D1 and cyclin E were significantly increased due to the overexpression of LAPTM4B-35, leading to accelerated G_1 phase progression and G_1/S transition. Taken together, our data support that LAPTM4B-35 promotes malignant proliferation via a mechanism that involves the activation of proto-oncogenes such as c-*myc*, c-*jun*, and c-*fos,* and subsequently the upregulation of some cell cycle regulators such as cyclin D1 and cyclin E.

Enhancement of migration and invasion are phenotypes of cell transformation and are involved in the process of metastasis of cancer cells. The migration and invasion of HLE-AE cells were significantly enhanced. These results are consistent with the fact that the expression of LAPTM4B-35 was relatively high in cell lines with high potential of metastasis, for example metastatic cell line PC-3M and BE1 cells express LAPTM4B-35 at higher levels than those non-metastatic cell lines PC3 and LH7 (both pairs of the cell lines were subcloned from the same prostate carcinoma cell line and pulmonary giant cell carcinoma cell line, respectively).[2] These data suggest that the overexpression of LAPTM4B-35 may play an important role in promoting the progression of cancer cells toward highly invasive and metastatic stages *in vivo*.

Cell proliferation, migration, and invasion are regulated by multiple signaling pathways. A PPRP motif, which serves as a recognition element and can interact with many SH3-containing signaling molecules,[32,33] is present in LAPTM4B-35 but not LAPTM4B-24. Our results in this study revealed that mutation of PPRP in LAPTM4B-35 significantly inhibited the malignant phenotypes of cancer cells, suggesting that LAPTM4B-35 may be involved in the regulation of certain signaling pathways. Mutation of the PPRP motif disrupts the association with its

interacting protein and, hence, leads to dysregulation of these pathways. Further studies into the details of these signaling pathways are being conducted in our laboratory.

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Abbreviations

APA	Acid phosphatase assay
BBS	Baby bovine serum
CDK	Cyclin-dependent kinase
FBS	Fetal bovine serum
HCC	Hepatocellular carcinoma
LAPTM4B	Lysosomal protein transmembrane 4 beta

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