Chapter 6

LAPTM4B Targeting as Potential Therapy for Hepatocellular Carcinoma

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Additional information is available at the end of the chapter

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Abstract

HCC is one of the most common cancers worldwide with high prevalence, recurrence, and lethality. The curative rate is not satisfactory. *LAPTM4B* is a novel driver gene of HCC first indentified by our group. It is over-expressed in 87.3% of HCC. The expression levels of the encoded LAPTM4B-35 protein in HCC is also over-expressed in 86.2% of HCC and shows a significant positive correlation with pathological grade, metastasis, and recurrence, and a negative correlation with postoperative overall- and cancer free-survival of HCC patients. Moreover, HCC cells showing high expression of LAPTM4B-35 show a strong tendency to metastasize and enhanced drug resistance. Overexpression of this gene promotes tumorigenesis, faster growth of human HCC xenografts and metastasis in nude mice, and leads to anti-apoptosis, deregulation of proliferation, enhancement of migration and invasion, as well as multi-drug resistance. In addition, overexpression of LAPTM4B-35 leads to accumulation of a number of oncoproteins and to down-regulation of a number of tumor suppressing proteins. By contrary, knockdown of endogenous LAPTM4B-35 via RNAi results in remarkable inhibition of xenograft growth and metastasis of human HCC in nude mice. Also, RNAi knockdown of LAPTN4B-35 can reverse the cellular and molecular malignant phenotypes noted above.

Therefore, it is suggested that to down-regulate over-expression of *LAPTM4B* gene and LAPTM4B-35 in HCC cells may provide novel strategy for HCC treatment. Moreover, the extensive effects caused by LAPTM4B-35 overexpression are based on its critical function in signaling network. Overexpression of LAPTM4B-35 activates at least 4 signaling pathways that are commonly known to be associated with tumorigenesis. Taken together, it is suggests that *LAPTM4B* is a HCC driver gene and LAPTM4B-35 is a key protein which functions in the upstream of cancer-associated signaling network and plays a critical role in tumorigenesis, progression, metastasis, multi-drug resistance and recurrence. Therefore, it may be worth considering the *LAPTM4B* gene and the LAPTM4B-35 protein a novel target in cancer therapy.

In recent years, we identified small chemicals that target LAPTM4B-35 for inhibiting HCC growth and metastasis. We screened 1697 chemicals and found ethylglyoxal bisthio-

semicarbazon (ETS) has effective anti-HCC activity probably via targeting LAPTM4B-35. Bel-7402 and HepG2 cell lines that highly express LAPTM4B-35 and a primarily cell line from naturally abortioned human fetal liver were used as the cell models and a control, respectively. Cell survival curve and apoptosis examination in vitro, and HCC xenograft growth and metastases in nude mice were measured to confirm the anti-HCC efficacy in vivo. Western blot, Co-IP, cDNA chips and RNAi were applied for mechanism study. The results showed that ETS can kill HCC cells but not human fetal liver cells *in vitro*, and also attenuate xenograft growth and metastasis of HCC and extend the life span of mice with HCC *in vivo*. When the endogenious over-expression of LAPTM4B-35 was knock-down by RNAi, the killing efficacy of ETS on HepG2 cells was significantly decreased. Also ETS inhibited the phosphorylation of LAPTM4B-35 Tyr_{285}, which involves in activation of PI3K/Akt signaling pathway induced by LAPTM4B-35 over-expression. In addition, all of the molecular alterations in HepG2 cells induced by LAPTM4B-35 over-expression can be reversed by ETS, including significantly decrease of c-Myc, Bcl-2 and phosphorylated Akt, but increase of Bax and phosphorylated p53. Accordingly, apoptosis was induced by ETS, and a number of pro-apoptotic genes were upregulated, while anti-apoptotic genes were downregulated. It is thus suggested that ETS may be a potential promising drug candidate for treatment of HCC by targeting LAPTM4B-35 protein.

In summary, our previous study demonstrated that *LAPTM4B* is a driver gene of HCC, targeting LAPTM4B may provide potential therapy for HCC. Targeting *LAPTM4B* includes bio-targeted therapy and chemical-targeted therapy. The bio-targeted therapy may further explore aimed at inhibiting over-expression of *LAPTM4B* gene via RNAi, miRNA or antisense RNA etc, as well as at blacking the functions of LAPTM4B-35 protein via specific antibody. The chemical-targeted therapy may further explore aimed at attenuating the over-activated signaling pathways in HCC by chemical inhibitors.

Keywords: LAPTM4B, Targeted HCC thrapy, ETS

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide with high prevalence, recurrence, and lethality. The curative rate is not satisfactory. Lysosomal protein transmembrane 4 beta (*LAPTM4B*) is a novel driver gene of HCC first cloned and indentified by our group [1,2]. *LAPTM4B* maps to chromosome 8q22.1 and encodes three isoforms of glycoprotein with four transmembrane regions, two extracellular domains (EC1 and EC2), and one small intracellular loop, together with both N-terminal and C-terminal tails, which reside in the cytoplasm. Three isoforms of LAPTM4B protein were designated as LAPTM4B-40, LAPTM4B-35, and LAPTM4B-24 according to their molecular weights [2]. Interestingly, overexpression of LAPTM4B-35 and LAPTM4B-24 show antagonist functions: LAPTM4B-35 promotes oncogenesis and the malignant cellular and molecular phenotypes, but LAPTM4B-24 promotes apoptosis and autophage [2].

LAPTM4B mRNA is overexpressed in 87.3% (48/55) of HCC by Northern blot analysis. The expression levels of the encoded protein LAPTM4B-35 is also over-expressed in 86.2% (T/N≥1.5 in 56/65) of HCC by Western blot analysis [4] and 71.8% (51/71) of HCC by immunohistochemistry [3] and show a significant positive correlation with pathological grade, metastasis,

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and recurrence and a negative correlation with postoperative survival of HCC patients [3-5]. Moreover, HCC cells with a high expression of LAPTM4B-35 show a strong tendency to motivate drug resistance [6]. The over-expression of this gene promotes tumorigenesis, faster growth, and metastasis of human HCC xenografts in nude mice [5,7] and leads to antiapoptosis, deregulation of proliferation, enhancement of migration and invasion, and multidrug resistance [5]. In addition, the overexpression of LAPTM4B-35 leads to the accumulation of a number of oncoproteins and downregulation of a number of tumor suppressing proteins. Conversely, knockdown of endogenous LAPTM4B-35 via RNAi results in remarkable inhibition of xenograft growth and metastasis of human HCC in nude mice [5,7]. Meanwhile, the RNAi knockdown of LAPTN4B-35 can reverse the cellular and molecular malignant phenotypes noted above [5]. It was also found in a number of solid cancers, including non-small cell lung cancer (NSCLC) that the level of LAPTM4B-35 expression was not only significantly higher than that in normal tissues and associated with histopathologic differentiation, lymph node metastasis, and TNM stage but also associated with microvessel density [8]. Taken together, it is suggested that *LAPTM4B* is a cancer driver gene and LAPTM4B-35 is a key oncoprotein, which are both predicted to be a diagnostic marker and a therapeutic target for cancer.

The extensive effects caused by LAPTM4B-35 overexpression are based on its critical function on cell trafficking and signaling network. Recently, Tan et al. [9] reported that the oncoprotein LAPTM4B not only interacts with EGFR but also regulates EGFR internalization and trafficking, and thus increases the amount and enhances the functions of EGFR on cell surface. Moreover, LAPTM4B can play a kinase-independent role for EGFR in autophagy initiation [10]. We found that the over-expression of LAPTM4B-35 can activate several signaling pathways that are commonly known to be associated with oncogenesis and progression [2]. The activation of PI3K/Akt signaling pathway induced by the overexpression of LAPTM4B-35 has been demonstrated to associate with drug resistance [6]. In this paper, we further present the functions of LAPTM4B-35 on signaling and a chemical that inhibits HCC *in vitro* and *in vivo* by targeting LAPTM4B-35.

2. Functions of LAPTM4B-35 involved in signaling network

Current evidence indicates that the interaction between cancer cells with their microenvironment plays key roles in oncogenesis and progression. Cancer microenvironment is composed of variant signal molecules, including solvable signal molecules (growth factors, cytokines, etc.), insolvable extracellular matrix (ECM), and variant cells nearby. Cancer cells and their microenvironment are reciprocally affected. Cancer cell proliferation, survival, and migrationare all motivated and dependent on not only solvable signal molecules but also ECM. Cancer cells accept positive or negative regulations of signal molecules from solvable factors, ECM, and other cells in their microenvironment through signal transduction pathways, which are organized as a very complicated network. In other words, cancer may be known as a disease of signaling network. Disturbances of signaling pathways and the converging network initiate at the early stage and go through the whole process of cancer development. In addition, the

disturbance of signaling pathways results from oncogenic alternation in genetics and epigenetics and contributes to the molecular and cellular malignant phenotypes of cancer cells, which include disregulations of proliferation, survival/apoptosis, differentiation and metabolism, as well as enhancement of migration/invasion and multidrug resistance. Therefore, signaling pathways and the network are of importance from a therapeutic perspective because targeting them may help reverse, delay, or prevent oncogenesis. Notably, since cirrhosis is associated with hepatic regeneration after tissue damages, which are caused by hepatitis infection, toxins (for example, alcohol oraflatoxin) or metabolic influences, and is often the prerequisite of hepato-oncogenesis, it is noticed that the ECM and the ECM-related signaling pathways, that are commonly alternated in cirrhosis and HCC, are of very importance. Our preliminary study has indicated that LAPTM4B-35 is most likely an assembly platform or organizer for a number of signaling molecules which are integrated in the cell membranes or soluble in the cytoplasm. Overexpression of LAPTM4B-35 would therefore be expected to lead to disturbance of a wide range of signaling pathways and their networks. We found LAPTM4B-35 can interact or co-localize with a number of these signal molecules, including membrane-integrated receptors and cytoplasmic signal molecules. These membrane-integrated receptors involve the growth factor receptors of the RTK (receptor tyrosine kinase) family, such as EGFR [9-11] and IGF-1R (Figure 1a), and ECM receptors of the integrin family, such as $\alpha 6\beta 1$ [11] and $\alpha 5\beta 1$ (Figures 1d and 2). The cytoplasmic signaling molecules that can interact with LAPTM4B-35 include FAK (Figure 2c) and PI3K p85α (Figure 3a). Given that LAPTM4B-35 is a tetra-transmembrane protein and localizes in plasma membrane and endomembranes (including lysosomes and endosomes). The interaction of LAPTM4B-35 with both RTK under the stimulation of growth factors, and integrin under ECM stimulation would be expected to integrate related signal transduction pathways triggered by growth factors and ECM components at the cell surface. It is well known that based on binding of growth factors (ligand) to their corresponding RTK receptor, Ras and ERK1/2 (MAPK family)downstream is subsequently activated [12]. At the same time, based on binding of ECM components (ligand), such as fibronectin (FN) or laminin (LN), to their corresponding integrin receptor ($\alpha 5\beta 1$ or $\alpha 6\beta 1$, respectively), FAK_{397} is phosphorylated and activated, and may subsequently activate downstream Ras/ERK and PI3K/AKT signaling pathways [13,14]. As has been previously recognized, the RTK/Ras/ERK signaling pathway and the ECM/Integrin/FAK signaling pathway converge at Ras and/or FAK. However, we found that over expression of LAPTM4B-35 can not only dramatically activate Ras (Figure 1b) and the downstream ERK1/2 (MAPK) under the stimulation of growth factors (Figures 1c) or FN (Figure 1f), respectively, but also activates FAK. This was originally suggested by knock down experiments. When LAPTM4B-35 is knocked down by RNAi in HCC cells, binding of integrin $\alpha 5$ with LAPTM4B-35 is dramatically decreased under stimulation with FN, as shown in Figure 1d. Knockdown of LAPTM4B-35 also coincidently significantly reduces phosphorylation and activation of FAK_{397} (Figure 1e) under stimulation by FN or LN. These experiments further provide evidence for the involvement of LAPTM4B-35 in the ECM/integrin/FAK signaling pathway. In addition, inhibition of FAK by PP2 (FAK inhibitor) can attenuate phosphorylation/activation of ERK1/2 in both LAPTM4B35-up-regulated HCC cells (AE) and in wild-type HCC control cells (Mock) as shown in Figure 1f. AE and Mock cells are both LAPTM4B-35 overexpressed, but to different extents. These results suggest that in LAPTM4B-35 overexpressed HCC cells, activation of ERK results from both the upstream growth factor/Ras and FN/

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Integrin/FAK signaling cascades. Taken together, it is reasonable to propose that overexpressed LAPTM4B-35 as a linker at the cell surface (plasma membrane) simultaneously over activates both the growth factor (EGF or IGF-1R)/RTK/Ras/ERK and the ECM (FN or LN)/Integrin/FAK/ERK signaling pathways by interacting with growth factor receptor (RTK) and ECM receptor (integrin) under the stimulation of growth factor and ECM components (FN, LN), respectively. In other words, the growth factor/RTK/Ras/ERK and ECM/integrin/FAK/ERK signaling pathways initiallyconverge at the plasma membrane level through overexpression of membrane-integrated LAPTM4B-35 in HCC cells, instead of at Ras and FAK in the cytoplasm in normal hepatocytes whichexpress LAPTM4B-35 and FAK at rather low level. Moreover, simultaneous overactivation of these two signaling pathways caused by LAPTM4B-35 overexpression would result in enhancement of proliferation, survival, migration and invasion of cancer cells.

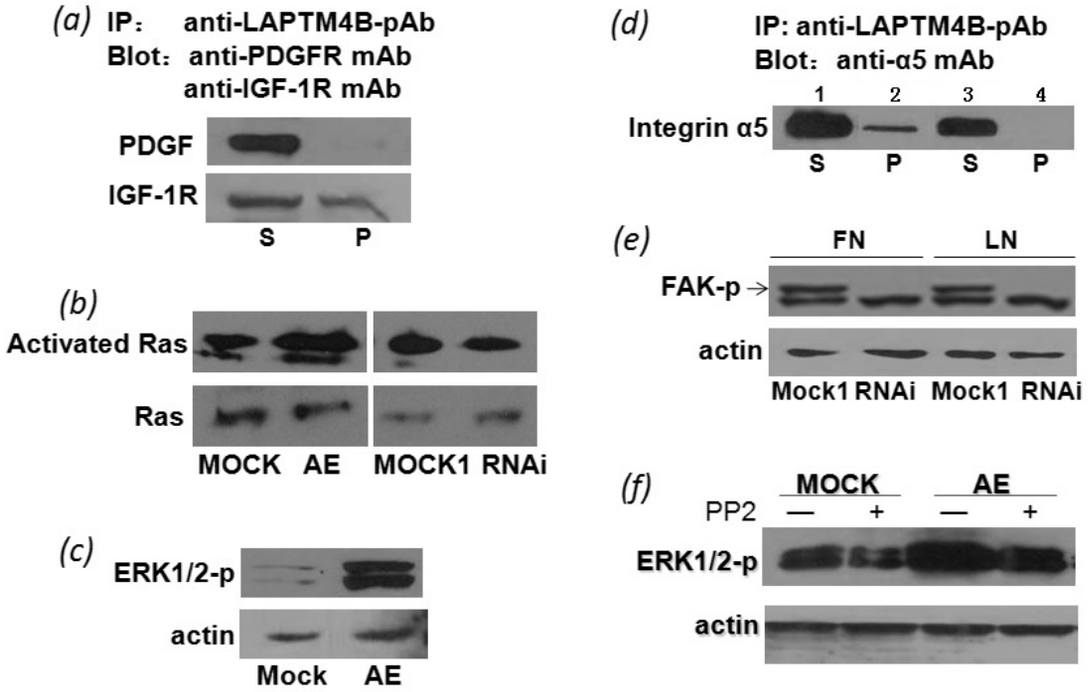

Figure 1. Activation of Ras/ERK and FAK/ERK signaling pathways by LAPTM4B-35 overexpression. *(a)* Co-IP assay indicates the interaction between LAPTM4B-35 and IGF-1R, but not PDGFR. Lysate from BEL-7402 HCC cells was immunoprecipitated by anti-LAPTM4B pAb, and the supernatant (S) and precipitant (P) were then subjected to Western blot with anti-PDGFR-mAb and anti-IGF-1R-mAb. *(b)* GST pull-down experiments with GST-RafRBD fusion protein to show Ras activation under stimulation of 20% fetal calf serum. The left panel indicates that activated Rasis increased inthe LAPTM4B-35 overexpressed BEL-7402 HCC cells (AE) as compared to the control cells (MOCK). The right panelindicates that activated Rasis decreased in the BEL-7402 HCC cells (RNAi)in which theLAPTM4B-35 has been knocked down via transient transfection by LAPTM4B-shRNA as compared with its control cells (MOCK1). It is obvious that activation of Ras in HCC cells is associated with overexpression of LAPTM4B-35. *(c)* Western blot analysis indicates that phosphorylated ERK1 and ERK 2 are increased in LAPTM4B-35 upregulated BEL-7402 HCC cells (AE) as compared with its control (MOCK) under stimulation of 20% fetal calf serum.*(d)* Co-IP assay indicates that the interaction between LAPTM4B-35 and integrin α5 and its dependent on the overexpression of LAPTM4B-35. The lysate of BEL-7402 HCC cells was immunoprecipitated with anti-LAPTM4B pAb, the supernatant (S) and precipitant (P) were then separately subjected to Western blot analysis with anti-integrin α5-mAb. In the Western blot profiles, Lanes 1 and 2 show the integrin α5 from the HCC MOCK1 cells (as a control) in the supernatant and immunoprecipitant, respec-

tively; Lanes 3 and 4 show the integrin α5 from LAPTM4B-35 knocked down (RNAi) HCC cells in the supernatant and immunoprecipitant, respectively. It is obvious, that integrin α5 in Lane 4 from LAPTM4B-immunoprecipitant of RNAi HCC cells Is dramatically reduced (disappear) as compared with Lane 2 from LAPTM4B-immunoprecipitant of wild-type HCC control cells that over express LAPTM4B-35. *(e)* Western blot analysis indicates that the phosphorylation/activation of FAK_{397} is reduced depending on knock down of LAPTM4B-35. The cells are stimulated by ECM component, either fibronectin (FN) or laminin (LN), for 15 min. The lysate of cells was then subjected to Western blot analysis. Anti- phosphorylated FAK_{397} mAb was used for blotting. The Western blot profiles indicate that based on stimulation of FN or LN, the phosphorylated FAK_{397} is reduced in cells which LAPTM4B-35 expression is knocked down as compared with the control cells.*(f)* Western blot analysis indicates that FAK inhibitor (PP2) inhibits the phosphorylation of ERK1/2. After treatment of BEL-7402 HCC cells (AE) by 1 μM PP2, the phosphorylation of ERK1/2 was analyzed via Western blot for the LAPTM4B-35 up-regulated cells and the MOCK cells under the stimulation of laminin substrate. The Western blot profile shows that phosphorylation/activation of ERK1/2 is associated with FAK activity.

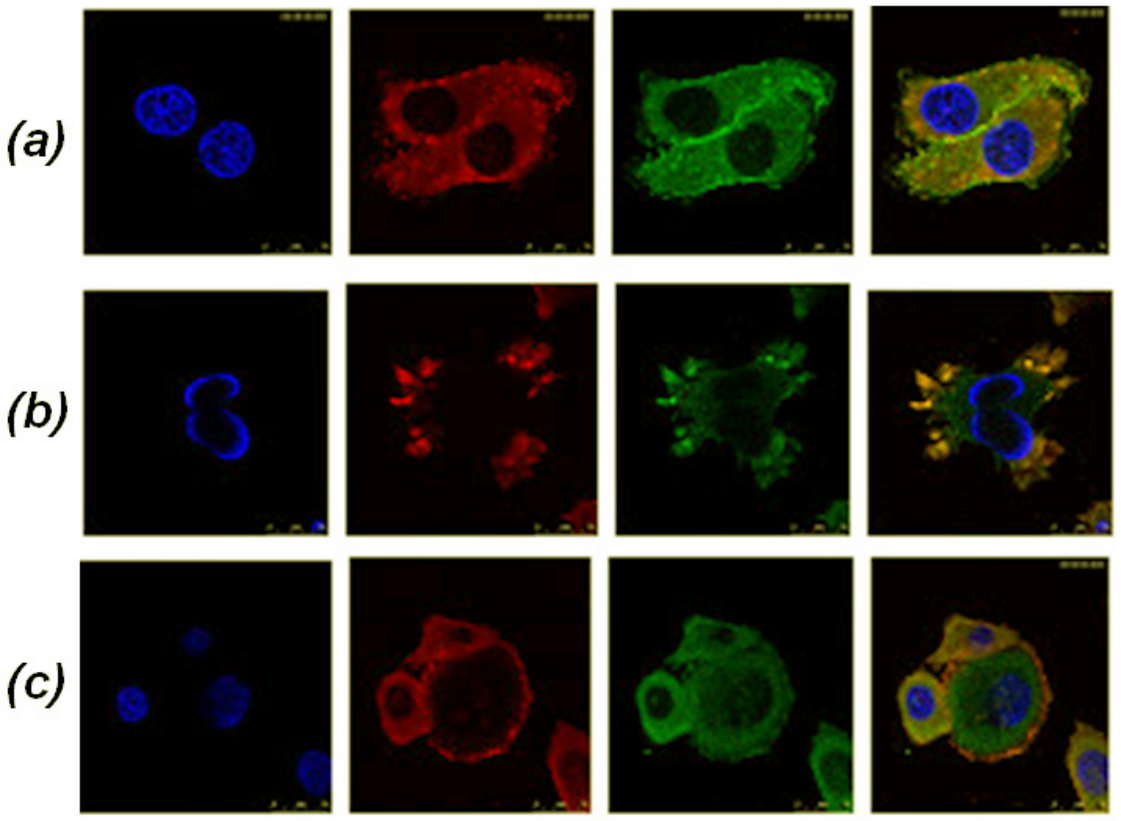

Figure 2. Colocalization between LAPTM4B-35 and integrinα5 or FAK. Cells were attaching and spreading onto fibronectin for 6 h *(a, c)* or 24 h*(b)*. *(a)* and *(b) show* the colocalization of LAPTM4B-35 (red) and integrin α5 (green). *(c)* shows the colocalization of LAPTM4B-35 (red) and FAK(green).

We found that not only membrane-integrated receptors, but also some solvable signaling molecules in cytoplasm can interact with LAPTM4B-35, such as FAK (Figure 2c) and PI3K p85α [6]. It is known that PI3K is a kinase which catalyzes phosphorylation of proteins andlipids. An important phosphorylated product catalyzed byPI3K is membrane-integrated PIP3 which can recruit cytoplasmic PH domain-containing proteins, including Akt and the corresponding kinases (PDK1 and PDK2) to the plasma membrane where Akt is phosphorylated by PDK1 and PDK2. Phosphorylated Akt is commonly known as a marker for PI3K/Akt

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signaling pathway activation. In view of the fact PI3K consists of two subunits: p110 catalytic subunit and p85α regulatory subunit. The kinase activity of p110 is normally inhibited by binding of p85α. The inhibitory effect of p85αcan be released by binding to an appropriate molecule [15]. We found that LAPTM4B-35 can interact with p85α, but not with p110 (Figure 3a). Moreover, using site-directed mutation experiments we found that binding of LAPTM4B-35 to PI3K p85α is mediated by two motifs. One is the proline-rich motif (PPRP) in the N-terminus of LAPTM4B-35, which may bind to the SH3 domain of PI3K p85αsubunit, and the other is phosphorylated Tyr_{285} in the C-terminus of LAPTM4B-35, which may bind to the SH2 domain of the PI3K p85α subunit (Figure 3b). To demonstrate this a series of HCC cell variants with highly expressed wild type and mutated LAPTM4B-35 were prepared by transfection with variant plasmids containing LAPTM4B-35 with mutation at PPRP or at Try $(Y)_{285}$, or with deleted N-terminus. These plasmids containg a FLAG sequence as a tag are designated as pcDNA3-LAPTM4B-flag (AF) containing wild type LAPTM4B-35, pcDNA3-LAPTM4B-flag (PA) containing P12,13,15A mutated LAPTM4B-35, pcDNA3-LAPTM4B-flag (△N) containing LAPTM4B-35 with a deletion of N_{10-19} amino acid residues), pcDNA3-LAPTM4B-flag (YF) containing Y285F mutated LAPTM4B-35, or pcDNA3-LAPTM4B-flag (△N+YF). As shown in Figure 3b, the binding of p85α to LAPTM4B-35 in HCC AF cells (up-regulated wild-type LAPTM4B-35) is dramatically increased under the stimulation of fetal calf serum, as compared with Mock cells (the control). In contrast, the binding of p85α to LAPTM4B-35 in the PA, △N, YF, and △N+YF-mutated HCC cell variants are all significantly attenuated under the same condition, as compared with AF cells. Therefore, the overexpression of LAPTM4B-35 in HCC cells would promote the interaction of both PPRP and Tyr-p motifs of LAPTM4B-35 with PI3K p85α and thus release the inhibitory effect of p85α regulatory subunit to the p110 catalytic subunit, and would cause the phosphorylation of the downstream AKT. Accordingly, Western blot analysis (Figure 3c) demonstrated that the phosphorylated Akt (Akt-p) is decreased in the mutated AF(PA) and AF(YF) cells as compared with the wild-type LAPTM4B-35 (AF), indicating that the proline-rich domain in the N-terminal and the Tyr_{285} in the C-terminal tails of LAPTM4B-35 are both required for Akt phosphorylation/activation. We also found that in the serum-starved HCC cells, LAPTM4B-35 and Akt separately distributes (Figure 4a); conversely under the stimulation of fetal calf serum which provides growth factors, co-localization of activated Akt and LAPTM4B-35 appears in the AF cells (Figure 4b); however, there is no co-localization in the PA-mutated cells (Figure 4c), YF-mutated cells (Figure 4d), and also in the cells in which PI3K is inhibited by its inhibitor LY294002 (Figure 4e). It is obvious that the co-localization of LAPTM4B-35 and Akt appears merely in cells wherein wild-type LAPTM4B-35 is up-regulated, but not in the cells transfected by the empty vector (Mock) nor in any of the cells with mutation of PA, ΔN, and YF of LAPTM4B-35. These results further provide evidence that the PI3K-dependent activation of Akt is associated with the up-regulation of LAPTM4B-35 expression via both proline-rich motif in the N-terminus and the Tyr-p in the C-terminus (Figure 5). It is therefore proposed that LAPTM4B-35 activates PI3K/Akt signaling pathway through binding PI3K p85α by a proline-rich domain at the N terminaus and a phosphorylated Tyr_{285} at the C terminus to release the inhibitory effect of p85α on PI3K p110 activity, and consequently result in phosphorylation and activation of Akt.

Moreover, we demponstrated that theTyr_{285} is the one single site for phosphorylation of Tyr residues in the LAPTM4B-35 molecule (Figure 3f). Notably, under stimulation of LN, Tyr_{285} phosphorylation rises quickly and peaks at 10 min. Thereafter phosphorylation decreases steadily out to 40 minutes (Figure 3g -1). It is of importance that LAPTM4B-35 Tyr_{285} phosphorylation can be markedly inhibited by LAPTM4B-EC2-pAb (Figure 3g -2), indicating the EC2 domain is required for Tyr_{285} phosphorylation of LAPTM4B-35. Kazarow (2002) reported that CD151, a member of the tetra-transmembrane protein family, can interact with the integrin α subunit via an QRD motif in the EC2 domain. Similarly, a YRD motif exists in the LAPTM4B-35 EC2 domain. We found that in LAPTM4B-35 $YRD_{233\text{-}235}$INF mutated HCC cells, AKT phosphorylation/activation is significantly inhibited (Figure 3h), suggesting interaction of LAPTM4B-35 EC2 YRD and integrin is involved in PI3K/AKT activation. In addition, LAPTM4B-EC2-pAb and integrin α6-mAb can both inhibit FAK phosphorylation under stimulation by LN (Figure 3i), indicating interaction of LAPTM4B-EC2 with integrin α6 (the specific receptor of LN) is involved in FAK phosphorylation/activation. Moreover, we found that the FAK inhibitor PP2 can simultaneously inhibit phosphorylation of LAPTM4B-35 and interaction of LAPTM4B-35 with PI3K p85α (Figure 3e). These result suggest that FAK is likely the kinase that catalyzes the Tyr phosphorylation of LAPTM4B-35, by which the binding site of LAPTM4B-35 to the PI3K p85α SH2 domain is created, thus releasing inhibition of PI3K p85α to p110 kinase activity, and consequently resulting in activation of downstream AKT (Figure 5).

It is known that FAK, as a functionally complicated signal molecule with Tyr kinase activity and nonkinase scaffolding function, is overexpressed in many cancers (including 60% of HCC) and involves in many aspects of tumor growth, invasion, and metastasis. Given that the phophorylation of FAK Tyr_{397} is critical for trigering its Tyr kinase activity and enhancing its nonkinase scaffolding function, and is induced by binding of integrin with FN or LN. We found that the PI3K/Akt signaling pathway in LAPTM4B-35 overexpressed HCC cells can be activated by stimulation of not only serum but also fibronectin or laminin substrate (Figure 3d); additionally the interaction of LAPTM4B-35 with PI3K p85α is inhibited by FAK inhibitor PP2 (Figure 3e). These results suggest that overexpression and interaction of LAPTM4B-35 and FAK in cancer cells would be expected tocreate an alternative signaling pathway, i.e. ECM/integrin/FAK/LAPTM4B-35/PI3K/AKT signaling pathway. In which FAK phosphorylation/activation results from interaction of the LAPTM4B-35 EC2 domain and integrin α6 subunit at the cell surface under the stimulation by LN or FN, and results in phosphorylation of LAPTM4B-35 Tyr_{285} by FAK kinase activity. This model (shown in Figure 5 on the upper right) illustrates a novel putative mechanism by which the PI3K/AKT signaling pathway is over activated through the involvement LAPTM4B-35 in cancer cells. In other words, our preliminary results suggest there might be a novel LAPTM4B-35 dependent pathway which gives rise to overactivation of the PI3K/AKT signaling pathway in HCC cells. In this mechanism, overexpressed LAPTM4B-35 interacts initially with integrin at the cell surface under stimulation of an ECM component (FN or LN) via its EC2 YRD motif. This interaction of LAPTM4B-35 and integrin induces phosphorylation and activation of FAK_{397} through a currently not fully understood mechanism. Activated FAK may catalyze phosphorylation of LAPTM4B-35 Tyr_{285} to create a binding site for PI3K p85α. Consequently, downstream AKT is phosphory-

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lated and activated by PI3K p110, the kinase activity of which comes into play through binding of phosphorylated LAPTM4B-35 Tyr_{285} to PI3K p85α. This proposed molecular mechanism remains to be further studied in detail.

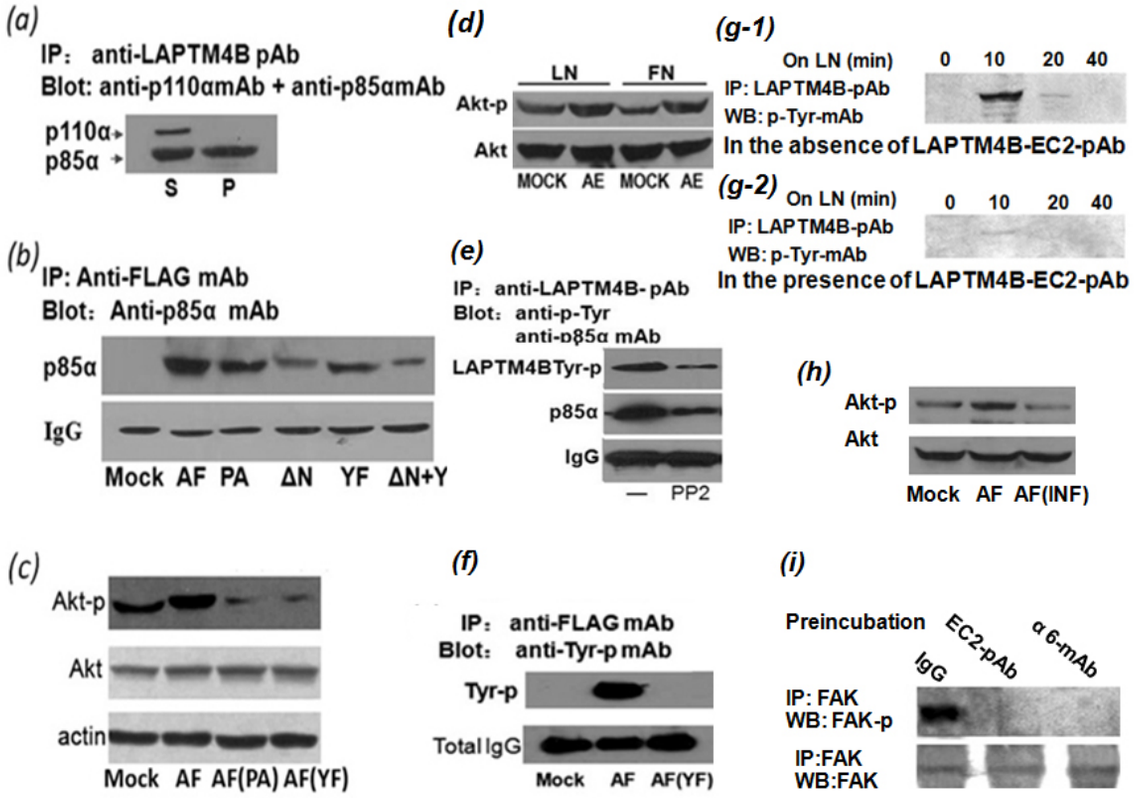

Figure 3. Mechanism for interaction of LAPTM4B-35 with of PI3K p85α and activation of Akt. *(a)* Co-IP analysis demonstrates interaction of LAPTM4B-35 with p85α regulatory subunit, but not PI3K p110 catalytic subunit. Anti-LAPTM4B35-pAb was used to precipitate the binding proteins, and a mixture of anti-PI3K p110-mAb and anti-PI3Kp85α-mAb was applied to blot the binding proteins. *(b)* Co-IP analysis demonstrates that the proline-rich domain in N-terminus and Tyr_{285} in C-terminus of LAPTM4B-35 are involved in the interaction of LAPTM4B-35 with PI3K p85α via a serious of mutants, including PA, $\triangle$N, YF, and $\triangle$N+YF mutants. PA mutant (P): Prolines in the PPRP motif in N-terminus of LAPTM4B-35 were mutated to alanines(P12,13,15A). $\triangle$N mutant: The 10th-19th amino acid residues in the N-terminus of LAPTM4B-35 were deleted. YF mutant: The Tyr_{285} in the C-terminus of LAPTM4B-35 was mutated to phenylalanine (Y285F). $\triangle$N+YF mutant: $\triangle$N mutant plus YF mutant. Anti-FLAG-mAb was used to immunoprecipitate the binding proteins in lysates from variant BEL-7402 HCC cell lines, which were transfected separately by pcDNA3-Mock-flag (Mock), pcDNA3-LAPTM4B-flag (AF), pcDNA3-LAPTM4B-flag (PA), pcDNA3-LAPTM4B-flag ($\triangle$N), pcDNA3-LAPTM4B-flag (YF), or pcDNA3-LAPTM4B-flag ($\triangle$N+YF) plasmids. Then anti-PI3Kp85α-mAb was applied to blot the binding proteins. The interaction of LAPTM4B-35 and PI3K p85α was dramatically enhanced in LAPTM4B-35 up-regulated AF cells as compared with the Mock cells and was significantly attenuated in the variant LAPTM4B-mutated cells as compared with the AF cells. *(c)* Western blot profile demonstrates that Akt-p is decreased in the mutated AF(PA) and AF(YF) cells as compared with wild-type LAPTM4B-35 (AF), indicating that the proline-rich domain in N-terminal and the Tyr_{285} in C-terminal tails of LAPTM4B-35 are necessary for Akt phosphorylation. *(d)* Western blot demonstrates that ECM components, fibronectin (FN) or laminin (LN), can promote phosphorylation/activation of Akt in cells in which LAPTM4B-35 expression is up-regulated, indicating association of phosphorylation/activation of Akt with FN and LN in HCC cells. *(e)* Co-IP analysis demonstrates that FAK inhibitor PP2 can simultaneously inhibit phosphorylation of Tyr_{285} and interaction of p85α with LAPTM4B-35. Anti-LAPTM4B-pAb was used to immunoprecipitate the binding proteins, then anti-phosphorylated Tyr mAb or anti-Akt mAb was used to blot the

binding protein. *(f)* Co-IP and Western blot profile show that LAPTM4B-35 Tyr_{285} is the only phosphorylation site by mutation analysis. HepG2 cells were transfected by AF or AF(YF) mutant. The phosphorylation appeared merely in the wild type HepG2 cells, but not the Tyr_{285} mutated YF cells. *(g)* Co-IP analysis indicates that LAPTM4B-35 Tyr can be phophorylated in a peaky manner under the stimulation of LN. HCC cells were placed on LN-coated vials for variant times, LAPTM4B-EC2-pAb was used to precipitate LAPTM4B protein in the HCC lysates. The immuno-precipitants were subjected to Western blot analysis. The anti-phosphorylated Tyr-mAb was used to blot the phosphorylated LAPTM4B-35. *(g-1)* shows the time course of LAPTM4B-35 phosphorylation with the highest phosphorylation at 10 min. *(g-2)* shows the inhibition of LAPTM4B-35 phosphorylation by LAPTM4B-EC2-pAb. *(h)* Western blot analysis indicates that mutation of YRD motif in EC2 domain of LAPTM4B-35 can inhibit AKT phosphorylation. BEL-7402 HCC cells were transfected by pcDNA3-AF(YRD233-235INF) mutated plasmids (INF) and the wild type pcDNA3-AF (AF) plasmids, respectively. The lysates were analyzed by Western blot with a anti-phosphorylated AKT-mAb. *(i)* Co-IP analysis indicates that both LAPTM4B-EC2-pAb and integrin $\alpha 6$ mAb can inhibit FAK phosphorylation. The BEL-7402 HCC cells were pre-incubated with non-immune IgG (as a control), LAPTM4B-EC2-pAb and integrin $\alpha 6$ mAb, respectively. The lysates were precipitated by FAK mAb. The immuno-precipitants were then subjected to Western blot analysis, and phosphorylated FAK mAb (the upper panel) or FAK-mAb (the lower panel) was used as the bloting antibody.

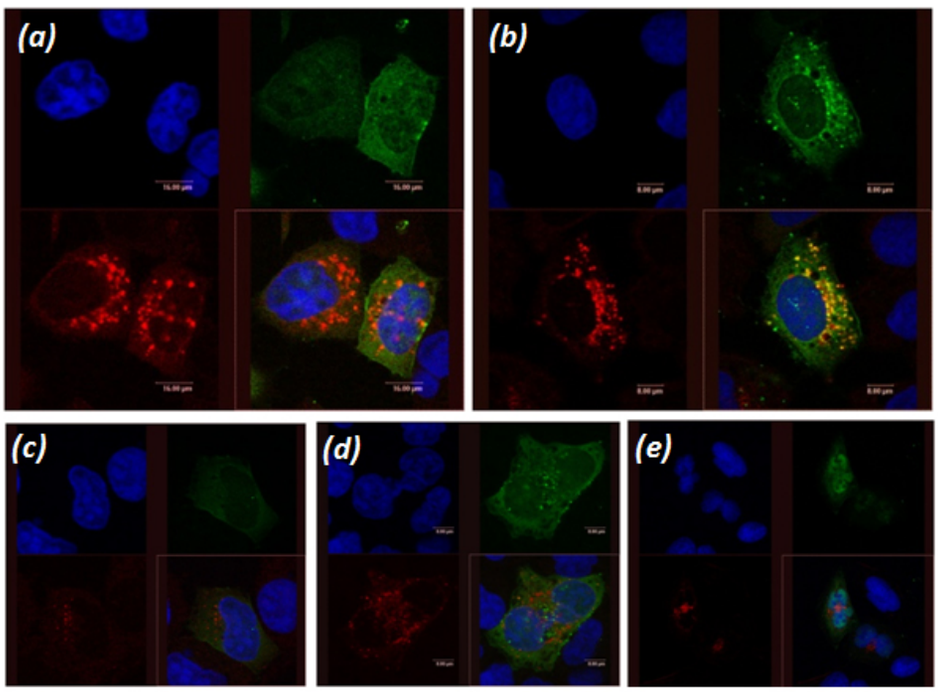

Figure 4. Co-localization of activated Akt and overexpressed LAPTM4B-35 under the stimulation of serum in BEL-7402 HCC cells. *(a)* Nonactivated Akt (green) and LAPTM4B-35 (red) are separately distributed in the cells cotransfected with pEGFP-PH-Akt plasmids and pcDNA3-LAPTM4B-flag plasmids (AF) after serum-starvation for 16 h. *(b)* Colocalization (yellow) of activated Akt (green) and overexpressed LAPTM4B-35 (red) understimulation of serum in HCC cells, which is stimulated by 20% fetal calf serum for 15 min after serum-starvation for 16 h. (c) No colocalization appeared in PA mutant HCC cells under the same conditions as described in *(b)*. (d) No colocalization appeared in YF mutant HCC cells under the same conditions as described in *(b)*. (e) No colocalization appeared in the presence of PI3K inhibitor (LY294002) in AF HCC (wild-type) cells under the same conditions as described in *(b)*.

In summary, cancer-targeted therapy currently focuses primarily on targeting key signaling molecules in one or more signaling pathways which are overactivated in a given cancer. Tetratransmembrane LAPTM4B-35 is believed to function as an assembly platform or organizer for a number of signaling molecules, which may either be integrated in the cell membranes or

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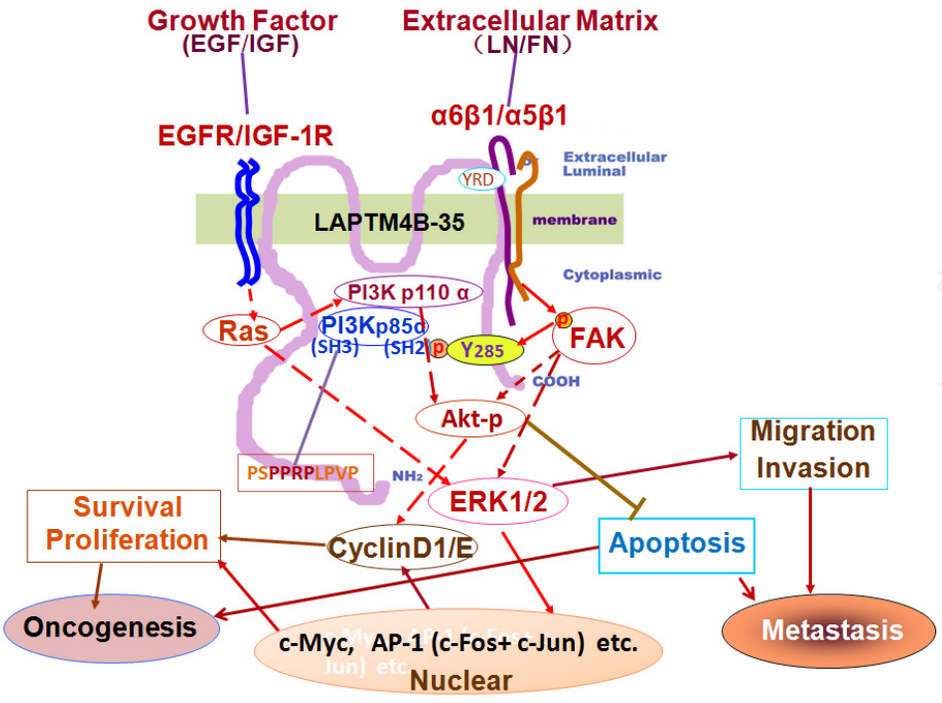

Figure 5. Signaling pathways activated by the overexpression of LAPTM4B-35 in HCC cells.

soluble in the cytoplasm. The LAPTM4B-35 overexpression, which occurs in more than 80% of HCC tissues, and the interactions with membrane-integrated receptors and cytoplasmic signal molecules are expected to act as an amplified assembly platform for upstream signal molecules of several signaling pathways, and leads to over activation of related signaling pathways (Figure 5), such as growth factor/ RTK/Ras/ERK, growth factor/RTK/Ras/PI3K/Akt, ECM/integrin/FAK/ERK, ECM/integrin/FAK/PI3K/Akt, and so on. Since these signaling pathways and their networks are closely associated with malignant molecular and cellular phenotypes, including cell proliferation/differentiation and survival/apoptosis as well as migration/invasion, it is believed that over activation of these signaling pathways is linked with hepatic carcinogenesis and progression [12-15]. Collectively, our data strongly suggest that LAPTM4B-35 would be an ideal target for HCC treatment, and that LAPTM4B-targeted therapy is a promising potential therapeutic strategy for HCC which will act in down regulation of the expression of LAPTM4B-35, or act by obstructing the interaction of LAPTM4B-35 with growth factors, integrins, FAK, PI3K p85*α* and other LAPTM4B-35 binding signal molecules.

3. Small chemicals targeting LAPTM4B-35

The molecular targets for cancer therapy have expanded from angiogenesis to oncogenic signaling pathways. The target indication has shifted from advanced stage to early or inter-

mediate stages of cancer. Agents targeting EGFR, FGFR, PI3K/Akt/mTOR, TGF-β, c-Met, MEK, IGF signaling, FAK and histone deacetylase have been actively explored [17,20].

Based on the basic characteristics: (1) *LAPTM4B* is a driver oncogene (2) this gene and theencoding LAPTM4B-35 protein are over expressed in more than 85% of HCC and (3) theoverexpression of LAPTM4B-35 can activate multiplesignaling pathways, we propose that *LAPTM4B* gene and the LAPTM4B-35 protein might bean ideal target for HCC treatment. We identified the chemicals that target LAPTM4B-35 for inhibiting HCC growth and metastasis. A total of 1697 synthetic small chemicals from Li and Liu (Pharmaceutical Institute, Chinese Academy of Medical Sciences) were screened. Among these chemicals, ethylglyoxal bis-thiosemicarbazone (ETS) was found to have effective activity for the inhibition of growth and metastasis of human HCC cells *in vitro* and *in vivo* probably via targeting LAPTM4B-35 [18].

Three HCC cell lines (Bel-74402, HepG2, and HLE) from human HCC and a cell line from naturally aborted human fetal were used as the cell models and a control, respectively. Cell survival curve and apoptosis analysis *in vitro* and HCC xenograft growth and metastasis in nude mice were evaluated to confirm the inhibitory efficacy *in vivo*. Western blot, Co-IP, cDNA chips, and RNAi were applied for exploration on mechanism.

We found that ETS can inhibit cell growth of variant HCC cell lines in a dose-dependent manner shown by cell growth curve *in vitro* (Figure 6a, 6b, and 6d). The IC50 of ETS inhibition varies for variant HCC cell lines, such as HepG2 (0.9 μmol/L), Bel-7402 (0.7 μmol/L), HLE (1.1 μmol/L), and H22 (1.6 μmol/L). Convesely, ETS cannot affect the survival of human fetal liver cells even if the concentration of ETS is increasing to as high as 200 times of that used for HCC cells. Notably, both Bel-7402 and HepG2 cells express LAPTM4B-35 at very high level and are most sensitive to ETS; HLE cells express LAPTM4B-35 at relatively low level [19] and are less sensitive to ETS. However, the fetal liver cells that express LAPTM4B-35 at a low level are not sensitive to ETS. Accordinly, when the endogenous overexpression of LAPTM4B-35 was knocked down by RNAi through shRNA transfection, the inhibitory effect of ETS on HepG2 cells was significantly decreased (Figure 6d). Figure 6c demonstrates the killing efficacy of ETS to HepG2 cell as shown by fluorescently double stained with Calcein-AM (1 μmol/L) and EthD-1 (2 μmol/L). Cells emitting green fluorescence were alive cells merely stained by Calcein-AM. Cells emitting red fluorescence were dead cells or apoptotic cells merely stained by EthD-1. Collectively, It is suggested that the inhibitory/killing efficacy of ETS on HCC cells depends on the high expression of LAPTM4B-35. At the same time, the effect of ETS on HepG2 cells was more effective than cisplatin (IC50: 7.5 μmol/L), doxorubicin (IC50: 7.6 μmol/L), mitomycin (IC50: 5.8 μmol/L), and 5-fluorouracil (IC50: >200 μmol/L) *in vitro* (Figure 6b). Moreover, the killing efficacy of ETS was confirmed from two aspects. First, after ETS treatment at a concentration of 1.25 μM for 72 h, HepG2 cells were cultured in a ETS-free medium at 37°C for as long as 12 days. As a result, when compared with 6×10^3 cells seeded in a well at the beginning, only a few colonies appeared after the12 days ETS-free culture, indicating that the vast majority of HepG2 cells were killed by ETS. Second, the significant killing efficacy of ETS on HepG2 cells was further confirmed by Calcein-AM/EthD-1 fluorescence double staining in a time-dependent manner (Figure 7b). The time-dependent growth inhibition was also shown by growth curves of HepG2 cells *in vitro* (Figure 7a) and HCC xenograft *in vivo* (Figure 8a).

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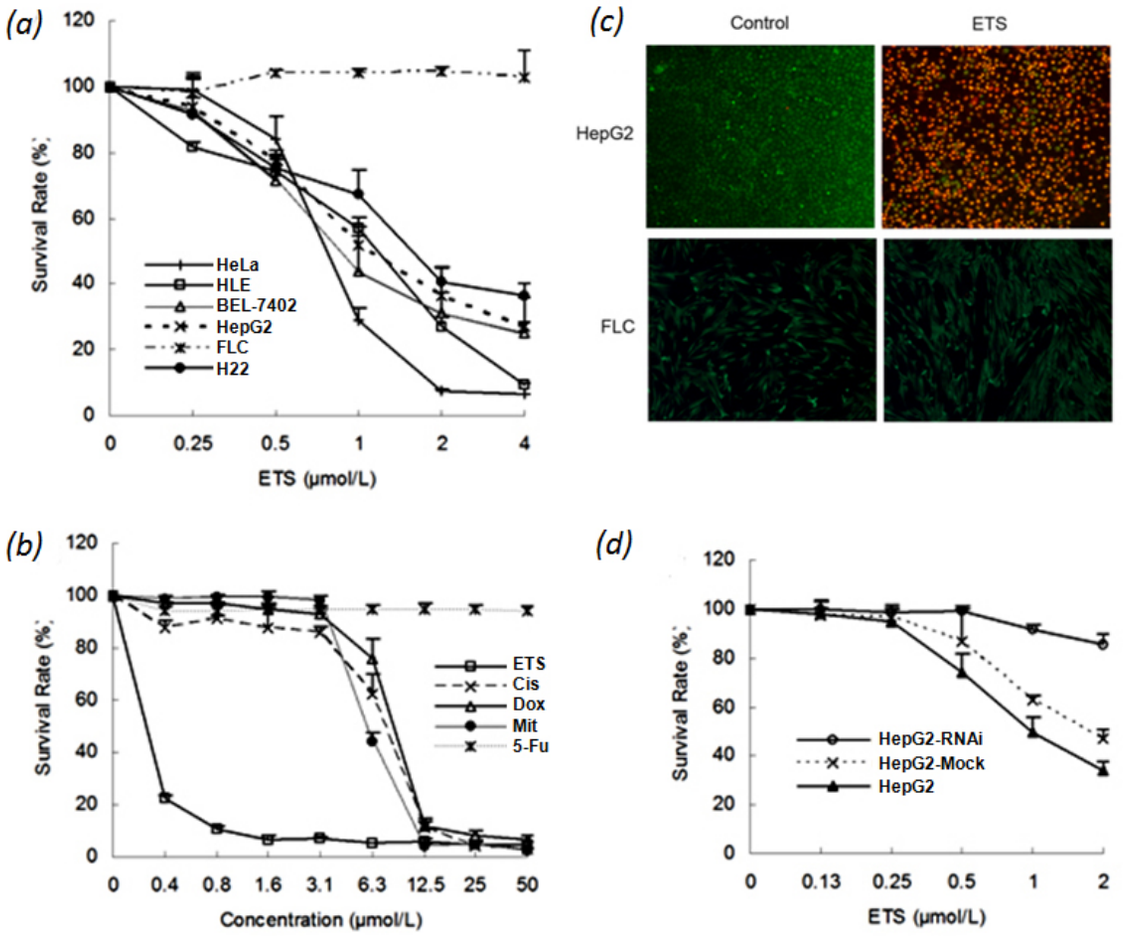

Figure 6. Inhibitory and killing efficiency of ETS on HCC cells. *(a)* Cancer cells of variant lines were incubated in the absence or presence of ETS at indicated concentrations for 48 h. *(b)* HepG2 cells were incubated in the absence or presence of variant drugs at indicated concentrations for 48 h. *(c)* The cells were fluorescently double-stained with Calcein-AM (1 μmol/L) and EthD-1 (2 μmol/L) at 37℃ for 30 min and then surveyed under fluorescence microscope. Cells emitting green fluorescence were alive cells which were merely stained by Calcein-AM. Cells emitting red fluorescence were dead cells or apoptotic cells which merely stained by EthD-1. Upper panel: HepG2 HCC cells were treated by ETS at a concentration of 2 μmol/L for 48 h. The vast majority of HepG2 cells were killed by ETS. Lower panel: human fetal liver cells were treated by ETS at a concentration of 25 μmol/L for 48 h. None of fetal liver cells were killed by ETS. *(d)* HepG2 cell line was transfected by LAPTM4B-shRNA or Mock. The transfected HepG2 cells by LAPTM4B-shRNA (RNAi) or LAPTM4B-Mock plasmids and the parent HepG2 cells were treated by ETS at indicated concentrations for 48 h. The LAPTM4B-35 silenced HepG2 cells showed less sensitive to ETS.The cell survival rate (%) of growth curves was calculated according to ratio of viable cells number determined by acid phosphatase assay (APA) before and after treatment.

ETS also shows significant effect on the inhibition of HCC growth and metastasis *in vivo*. Human HCC BEL-7402 cells were subcutaneously inoculated, and then ETS was administered either by intratumor injection or intraperitoneal injection. Both ways can inhibit the HCC xenograft growth. The effect of ETS on attenuation of growth and metastasis of human HCC xenograft in nude mice is shown in Table 1, as well as Figure 8(a) and 8(b). At the same time, the mice treated by ETS were less lost their body weight than that treated by mitomycin and

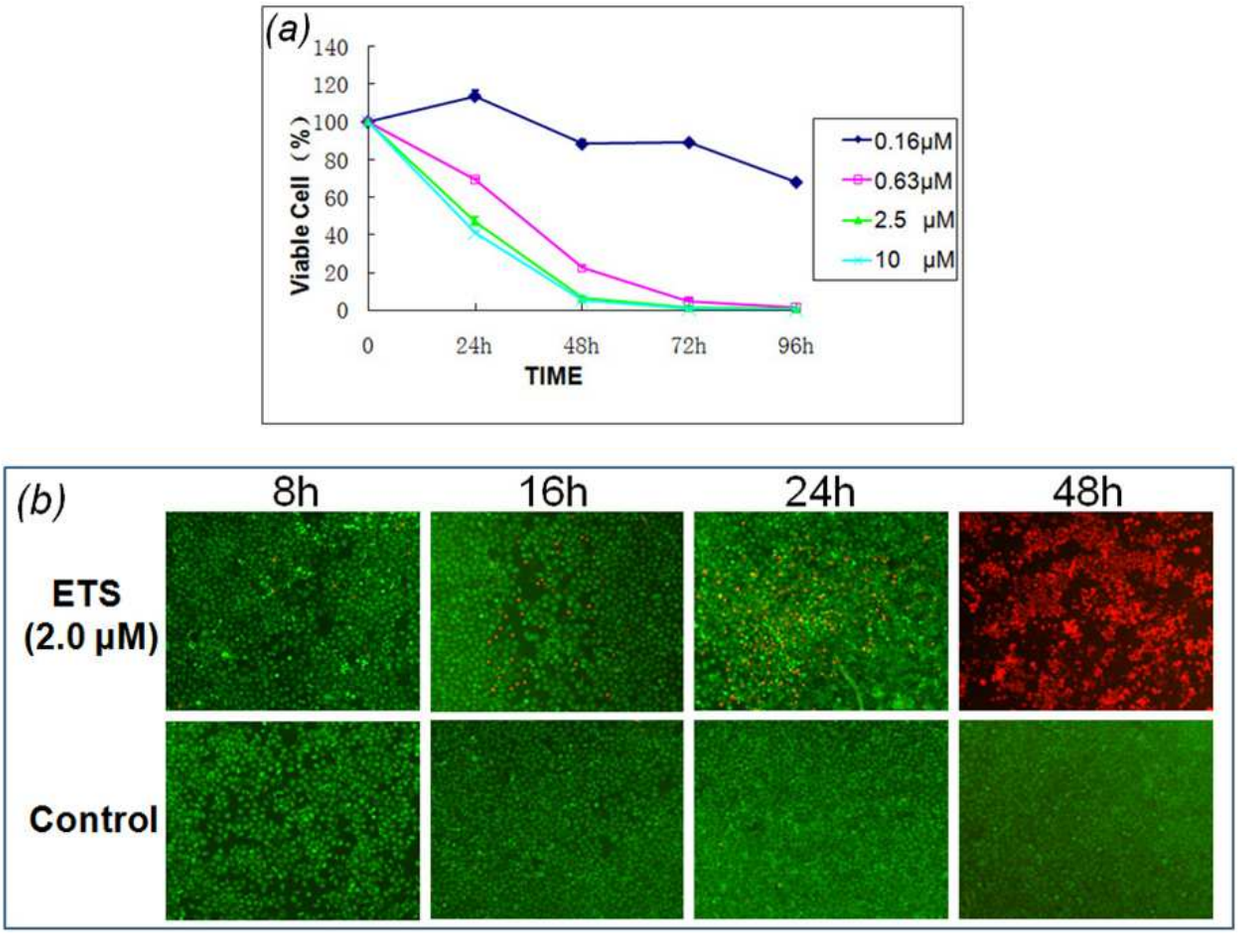

Figure 7. The time- and dose-dependent inhibition and killing of ETS on HepG2 cells. *(a)* HepG2 cells were treated with ETS at variant concentrations or for variant hours. The number of viable cells was determined by ASA. This figure shows that the effect of ETS on inhibiting HCC cell growth is dose and time dependent. *(b)* HepG2 cells were treated as Figure 6(c). Upper panel: with ETS (2 μM) for indicated incubation time. Lower panel: without ETS for comparable incubation time as a control. This figure shows that the effect of ETS on killing HCC cells is time-dependent.

cisplatin. As a matter of fact, the acute toxicity test indicated that ETS had little poison on mice. There is no death of mice in the 1000mg/kg, 464mg/kg, and control groups. Of the 10 mice per group, all died in the 4640mg/kg group, and 4 mice died in the 2150mg/kg group. The LD_{50} of ETS was 2329.9 mg/kg, with a 95% dependable limit of 1846.7-2939.0 mg/kg.

In addition, a murine HCC H22 cell line was applied to study the effect of ETS on the life span of mice with ascetic HCC. A dose-dependent prolongation of life span was observed as shown in Figure 8(c).

To illustrate the mechanism for killing HCC cells of ETS, apoptosis was studied at cellular, molecular, and gene levels. Flow cytometry showed that ETS (2μmol/L) can induce apoptosis of HepG2 cells in a time-dependent manner, i.e., 10.1% (8 h), 15.8% (16 h), 29.1% (24 h), 63.0% (36 h), and ~100% (48 h). The apoptotic cell rate includes all apoptotic cells at early and late apoptotic phases. Western blot analysis showed that along with the prolonged time of ETS treatment, the antiapoptotic Bcl-2 is decreasing and proapoptotic Bax is increasing (Figure

Group	No. of mice	Tumor size (X ± S), cm^3	Inhibitory rate (%)	Tumor growth rate	Metastasis of lymph node (number) (X ± S)
PBS control	8	1.96 ± 0.133	0	100%	3.3 ± 0.89
Solvent control	8	2.073 ± 0.118	0	100%	3.5 ± 1.07
ETS (5 mg/kg)	8	1.276 ± 0.104*	38.4%	58.5%	2.8 ± 0.71
ETS (15 mg/kg)	8	0.794 ± 0.090*	61.7%	52.6%	1.8 ± 0.71
ETS (45 mg/kg)	8	0.485 ± 0.123**	76.6%	31.7%	0.8 ± 0.71
Mitomycin (2 mg/kg)	8	0.673 ± 0.119**	67.5%	38.9%	0.9 ± 0.83
Cisplatin (2 mg/kg)	8	0.734 ± 0.098**	64.6%	41.9%	1.0 ± 0.76

*$p < 0.05$ vs. controls.

**$p < 0.01$ vs. controls.

Table 1. Inhibitory efficacy of ETS on the xenograph of human HCC in nude mice

9a). Notably, the phosphorylation of p53 protein is also increasing, suggesting that ETS might stabilize p53 protein, the key apoptosis regulator. Western blot analysis also showed that the key effecter molecule of apoptosis pathway, caspase 3, was activated from procaspase into cleaved caspase by ETS in a time-dependent manner (Figure 9c). At the same time, cDNA array analysis showed that a large number of proapoptotic genes were up-regulated and a large number of antiapoptotic genes were down-regulated by ETS treatment (Figure 9d).

Based on LAPTM4B-35 overexpression in HCC can up-regulate a number of oncogenes that promote cell proliferation and/or resist apoptosis, the effects of ETS on the expression of oncoproteins were detected. We found that all the molecular alterations in HepG2 cells induced by LAPTM4B-35 overexpression can be reversed by ETS (Figures 9-11), such as significant decrease of c-Myc (Figure 9b), cyclinD1, and Bcl-2 (Figure 9a) but increase of Bax and phosphorylated p53 (Figure 9a).

It is well known that PI3K/Akt signaling pathway plays a key role in antiapoptosis and cell survival in a large number of cancers and thus is considered as a target for cancer therapy [20]. We have found that the PI3K/Akt/GSK3β signaling pathway is overactivated by LAPTM4B-35 overexpression [5,6]. The effect of ETS on PI3K/Akt signaling was detected.We found that the phosphorylated Akt (Akt-p) is significantly reduced in the ETS-treated HCC cells either in the presence or absence of serum stimulation (Figure 10a). Then the mechanism was explored. Co-IP and Western blot analyses showed that ETS significantly decreased the phosphorylation of LAPTM4B-35 Tyr_{285} in C-terminus of LAPTM4B-35 (Figure 10b) and therefore the activation of PI3K/Akt signaling pathway is minimized via reducing interaction of LAPTM4B-35 and PI3K p85α (Figure 12).

In summary, our previous study demonstrated that *LAPTM4B* is a driver gene of HCC, and LAPTM4B-35 targeting may provide potential therapy for HCC. To target LAPTM4B for cancer therapy includes bio-targeted therapy and chemical-targeted therapy. The bio-targeted therapy may further explore aimed at inhibiting the overexpression of *LAPTM4B* gene via RNAi, miRNA, or antisense RNA, etc., as well as at blocking the functions of LAPTM4B-35

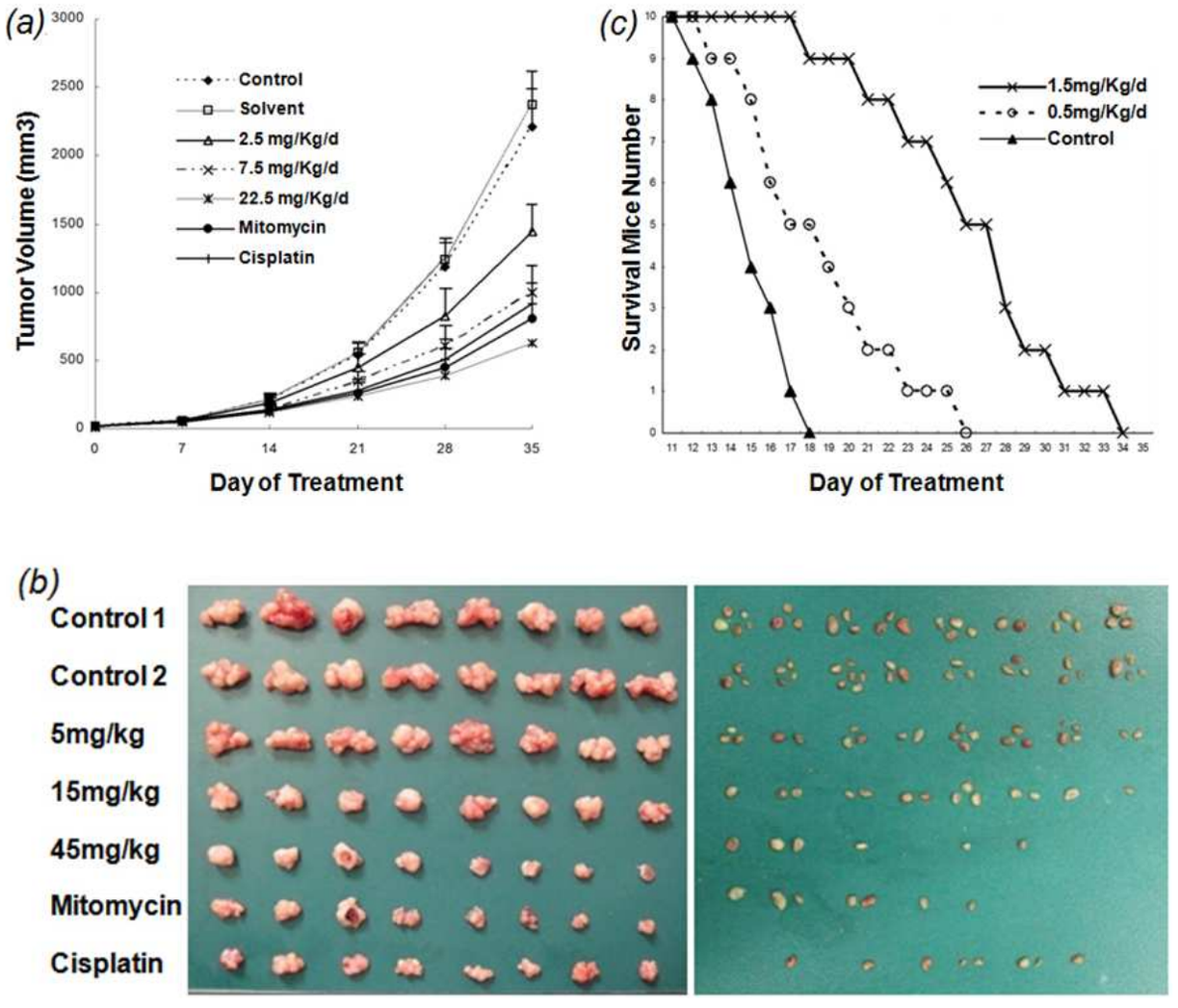

Figure 8. Inhibitory effect of ETS on growth and metastasis of human HCC xenograft in nude mice. Human HCC Bel-7402 cells (1×10^6) were inoculated into each nude mice. ETS (5, 15, or 45 mg/kg), cisplatin (2.0 mg/kg), mitomycin (2.0 mg/kg), PBS (control 1), or solvent (control 2) was administered every other day for each BALB\c-nude mouse in variant groups ($n = 8$), respectively, by intraperitoneal injection from day 9 when the xenograft grew out. Tumor volume was measured twice a week. The inhibitory efficacy on xenograft growth of ETS was observed to be dose-dependent as compared with the control groups of solvent and PBS. Mitomycin and cisplatin were used as the positive controls. *(a)* Tumor growth curves of human HCC xenograft in nude mice with variant treatments. *(b)* Tumor photograph of human HCC xenograft in nude mice with variant treatment for 6 weeks. Left panel: Size of human HCC xenografts in variant groups. Right panel: Number of lymph node metastases in variant groups. *(c)* The survival curves of mice with ascetic HCC in variant groups. Mouse hepatocellular carcinoma H22 cells (1×10^6) were inoculated into peritoneal of each ICR mouse. ETS (0.5 or 1.5 mg/kg) or the solvent was intraperitoneally administered every other day for each ICR mouse in variant groups ($n = 10$). The life span showed a significant prolongation in the ETS groups in a dose-dependent manner.

protein via specific antibody. The chemical-targeted therapy may further explore aimed at attenuating the overactivated signaling pathways by chemical inhibitors and thus inhibiting proliferation and inducing apoptosis. More signaling pathways and more complicated signaling network are supposed to be involved in deregulation induced by LAPTM4B-35 overexpression in cancer. Thus, the mechanism of ETS for targeting LAPTM4B-35 may be more complicated.

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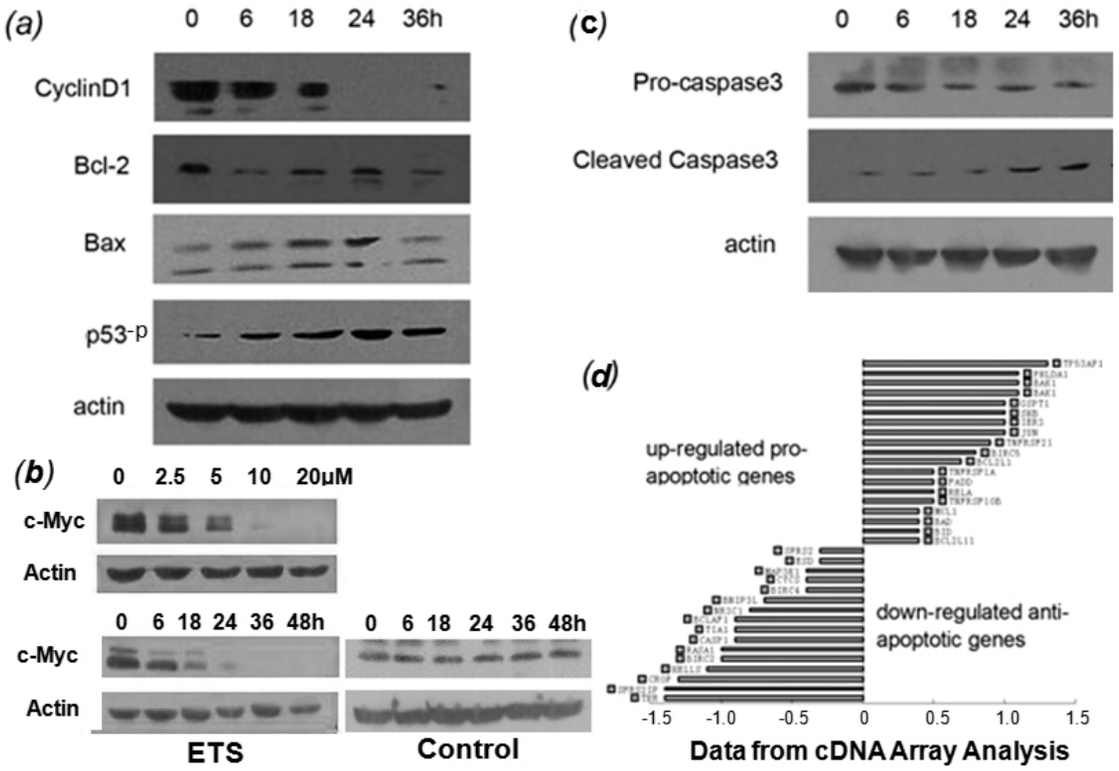

Figure 9. Apoptosis-related molecular alteration induced by ETS. *(a)* Western blot profiles of cyclin D1, Bcl-2, Bax, and phosphorylated p53 proteins from lysates of HepG2 cells incubated in the presence of ETS (2 μM) for indicated times, indicating that proliferation- and apoptosis-related proteins are altered by ETS in a time-dependent manner. *(b)* Western blot profile of cMyc protein from lysates of HepG2 cells incubated in the presence of ETS at indicated concentration for indicated hours, indicating remarkable decrease of c-Myc protein by treatment of ETS in a dose- and time-dependent manner. *(c)* Western blot profile of procaspase 3 and cleaved caspase 3 from lysates of HepG2 cells incubated in the presence of ETS (2 μM) for indicated times, indicating the activation of key effecter molecule in apoptotic pathway by ETS. *(d)* cDNA array analysis shows the up-regulated and down-regulated genes that promote and inhibit apoptosis, respectively, by treatment of ETS.

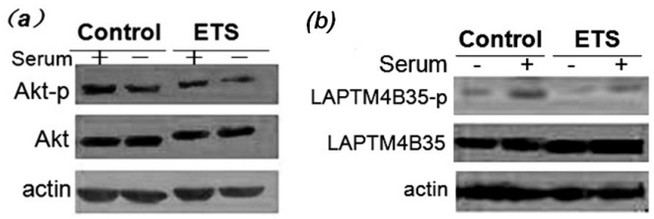

Figure 10. Inhibitory effects of ETS on phosphorylation of Akt and LAPTM4B-35. *(a)* Western blot profile of phosphorylated Akt from lysates of HepG2 cells incubated in the absence and presence of ETS (2 μM), indicating the inhibitory effect of ETS on activation of PI3K/Akt signaling pathway under stimulation with and without serum. *(b)* Co-IP and Western blot profile shows that ETS significantly decreased the phosphorylation of Tyr of LAPTM4B-35 protein. HepG2 cells were first serum-starved for 16 h, then serum and ETS or PBS (control) were added for 15min. The cell lysate was first precipitated by anti-LAPTM4B-N10-pAb, which reacts with LAPTM4B-35. After absorption by protein G/A agarose beads, the precipitant was subjected to Western blot analysis with antiphosphorylated Tyr-mAb. The profile shows that compared with the control, the phosphorylated LAPTM4B-35 is attenuated by ETS treatment in either presence or absence of serum stimulation.

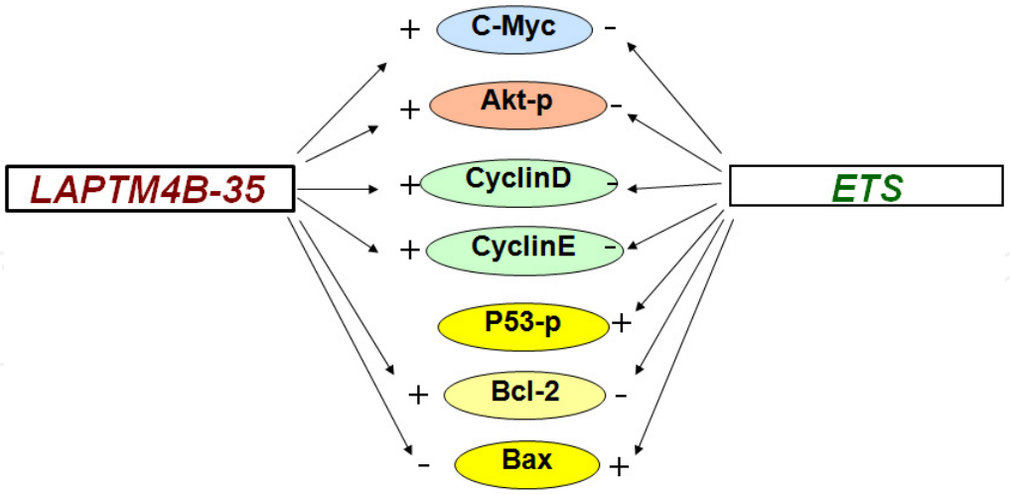

Figure 11. The antagonistic effects of ETS vs LAPTM4B-35 overexpression on expression of oncogenes and tumor suppressor genes in HCC.

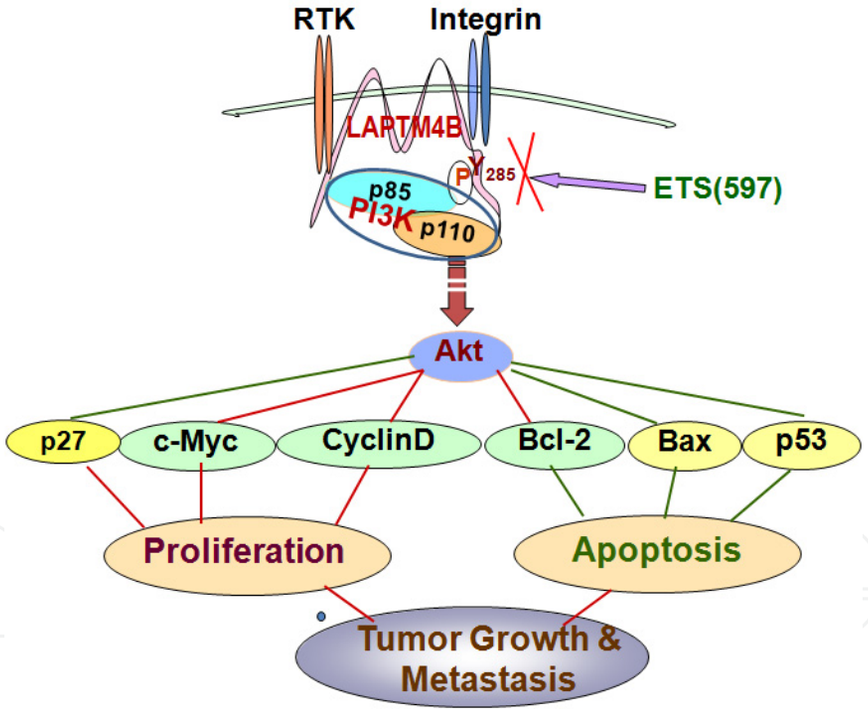

Figure 12. Molecular mechanism of ETS for targeting LAPTM4B-35.

4. Conclusion

Given that *LAPTM4B* is a driver gene of HCC and the encoding LAPTM4B-35 protein is overexpressed in HCC and contributes to the cellular and molecular malignant phenotypes [2],

the study on molecular mechanism reveals that the overexpression in HCC of the membrane integrated LAPTM4B-35 functions as an amplified assembly platform or organizer of related signaling molecules that are either integrated in cell membranes or solvable in cytoplasm, and thus activates several signaling pathways, such as growth factor/ RTK/Ras/ERK (MAPK), growth factor/RTK/Ras/PI3K/Akt, ECM/integrin/FAK/ERK (MAPK) or ECM/integrin/FAK/ PI3K/Akt, etc. Therefore, it is worth considering the *LAPTM4B* gene and the LAPTM4B-35 protein as novel targets in HCC therapy. A small chemical (ETS) can inhibit HCC cell growth and induce apoptosis *in vitro*, and inhibit growth and metastasis of human HCC xenograft *in vivo*. Notably, ETS can reverse the molecular alterations, that are induced by LAPTM4B-35 overexpression and involved in promotion of proliferation and survival of cancer cells. Moreover, ETS inhibits the phosphorylation of LAPTM4B-35 Tyr_{285}, a key motif forbinding to PI3K p85α regulatory subunit,, and thus inhibits the PI3K/Akt signaling pathway. Taken together, developing strategies for LAPTM4B-35 targeting can be a potential treatment for hepatocellular carcinoma therapy.

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