Organellar Proteomics Identifies Mouse Liver Nuclear Proteins

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Four fractions from mouse liver by the classical cell fraction: crude nuclei (CN), primary purified nuclei (PNN), purified nuclei (PN), total liver (TL), were analyzed by RP-HPLC-ESI-MS/MS on an LTQ linear ion trap mass spectrometer. The nuclear marker proteins analysis, the Swiss-Prot annotation and the bioinformatic prediction results proved that the nuclear proteins were enriched in the PN fraction. A total of 1348 mouse proteins were identified and 680 mouse proteins in the PN fraction. We combined the results of the Swiss-Prot annotation and the bioinformatic prediction to produce a list of 277 mouse nuclear proteins, including 98 proteins not previously associated with this organelle. Combination of subcellular fractionation with RP-HPLC-ESI-MS/MS on an LTQ linear ion trap mass spectrometer was proved to be a high-throughput, sensitive and effective analytical approach for subcellular proteomics research.

Proteomic Studies on Hepatitis C Virus-related Human Hepatocellular Carcinoma

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Chronic infection with hepatitis C virus (HCV) is one of the most clearly established risk factor for tumorogenesis of human hepatocellular carcinoma (HCC). The number of HCV-carriers in Japan is estimated to be 2 million and 32,000 of HCC patients passed away every year. The incidence of HCC is increasing in many countries in parallel to the increase of HCV infection. Therefore, it is urgent task to elucidate the mechanism involved in hepatocarcinogenesis by chronic HCV infection. We performed transcriptomic and proteomic studies using samples of tumor tissues and non-tumor tissues from HCC patients with HCV infection but not hepatitis B virus infection. The transcriptomic study using oligonucleotide DNA microarray indicated that GST gene responsible for detoxification and C15, IP27, C6 and OAS1 genes for immune response were up-regulated both ADH and CYP genes for detoxification were down-regulated in tumor tissues in the comparison of non-tumor tissues. The proteomic study using two-dimensional gel electrophoresis with MALDI-TOF-MS and LC/MS/MS showed that HSP 70 family such as GRP78, HSC70, GRP75 and HSP70.1, glutamine synthetase isoforms, alpha-enolase, phosphoglycerate mutase, and triosephosphate isomerase were increased but albumin, ferritin light chain, smoothelin, tropomyosin beta chain, arginase 1, aldolase B and ketohexokinase were decreased in the tumor tissues. We will discuss on the mechanism of HCV-related hepatocarcinogenesis and the potential use of proteomic profiling for finding biomarkers of HCC.

Exploration of Autoantibodies in Rat Orthotopic Liver Transplantation by Functional Proteomic Analysis

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Orthotopic liver transplants (OLT) performed in certain combinations of donor and recipient rat strains, such as DA (RT1a) to PVG (RT1c), without immunosuppressive drugs could completely overcome major histocompatibility complex barriers. Although other organs transplanted in a similar fashion within the same combination have been promptly rejected, the 60 day post-OLT serum (POD 60) has been proven competent in rapidly reversing the established rejection in animal models. However, there have been several possible mechanisms reported, the properties responsible for the tolerogenic phenomenon is not fully understood. There is increasing evidence for an immune response to transplant in humans or rodents, demonstrated in part by the identification of autoantibodies to transplant rejector or tolerogenic antigens. The identification of panels of rejection or tolerogenic antigens that elicit a humoral response may have utility in rejection screening, diagnosis, or in establishing transplant tolerance without immunosuppressive drug.

Proteomic approach we have utilized allows identification of autoantibodies to proteins in lysates prepared from naive or transplanted livers and tumor cell lines and thus may readily uncover antigenicity associated with post-translational modification which involved rejection or tolerance establishment in spontaneous liver transplantation. These will provide guidance with respect to discovering potential protein targets in OLT tolerance and eventually prolong hepatic allograft survival in the future.

P53 Mediated Cellular Pathways: Potential Role in HCV Associated Liver Injury

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Number of cellular pathways related to the apoptotic process involved in chronic HCV hepatitis have been studied. However, no data on the expression of p53 is being available that plays a dual role in response to tissue damage, either by inducing growth arrest and/or apoptosis. Recent studies including our research have shown G1 arrested hepatocytes in chronic HCV patient biopsies. Here in continuation to our previous research we focuses on how induction of G1 arrest and induction of apoptosis are related to one another, for HCV infection associated hepato-cellular injury. P53 being an important candidate for this position was examined immunohistochemically in forty six HCV positive patient’s liver biopsies (Stage 0/II/III/IV, 09/10/13/07/07). The expression was correlated with either p21 (cyclin dependent kinase inhibitor) or TUNEL staining. Increased expression of p53 and p21 was observed in sections from chronic HCV patients and both were significantly correlated with the stage of fibrosis. Significant correlation with TUNEL staining was also observed at fibrotic stage 2–4. Thus, these findings suggest possible involvement of p53-dependent pathways in the growth and apoptosis of hepatocytes during the pathogenesis of HCV infection. However, the mechanism of p53 activation remains to be identified.
9.5 Comparative Proteome Studies of Nuclear Proteins in Hepatocellular Carcinoma Cell Lines with Different Metastatic Potential

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The most common primary cancer of the liver is hepatocellular carcinoma (HCC). It is responsible for approximately one million deaths each year, mainly in underdeveloped and developing countries. The bottleneck in the treatment is the recurrence and metastasis of the carcinoma cell. The nucleus contains the genetic information and is the place of gene expression. In our experiment, we extracted the nuclear proteins through differential velocity centrifuge. The nuclear proteins were separated by 2-D gel electrophoresis. After the image analysis, we found some differentially expressed nuclear proteins in HCC cell lines with different metastatic potential (Hep3B, MHCC97-L, LM3). 85 protein spots were found to increase/decrease more than 3 fold between the Hep3B (a cell line with HCC but no metastatic potential) and MHCC97-L (a cell line with HCC and low metastatic potential), and 61 spots were found to increase/decrease more than 3 fold between the MHCC97-L and LM3 (a cell line with HCC and high metastatic potential). These spots were identified by the MALDI-TOF-TOF MS after in gel tryptic digestion. Totally 116 of the 146 spots were successful analyzed, corresponding to 93 gene products. Most of the identified proteins were proved to locate in the nucleus. We found proteins such as CK8, 18, 20, LaminA/C, Lamin B, hnRNP C, and hnRNP H have different expressions among the 3 cell lines. Some of them have been selected for the following functional research.

9.6 Proteomic Analysis on Metastasis-associated Proteins of Hepatocellular Carcinoma Tissue

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Hepatocellular carcinoma (HCC) is the 3rd most common cause of death from cancer worldwide. Metastasis remains one of the major challenges for conquering the malignancy finally. In this study, a proteome approach was used to identify and analyze proteins that were relevant to metastasis of HCC. Proteins extracted from 12 liver tumor tissue specimens (6 with metastases and 6 without) were separated by two dimensional gel electrophoresis (2-DE). Comparative analyses of 2-DE protein patterns between the two groups were done using computerized image analysis and naked eyes. Selected proteins exhibiting statistically alternations were identified by mass spectrometry. Western blotting and RT-PCR were performed to examine expression of candidate proteins. 16 proteins including HSP27, S100A11, GST were annotated by mass spectrometry, which were relative to cell mobility, signal transduction, energy metabolism and so on respectively. Of these HSP27 was found to uniquely over-expressed in 2-DE maps of all 6 metastatic HCC when compared to the non-metastatic HCC tissues. Western blotting and RT-PCR of other 6 pair of HCC tissues confirmed this difference. This study suggested that various different proteins join together in HCC metastasis. The overexpression of HSP27 may serve as biomarkers for early detection for therapeutic targets unique to the metastatic phenotype of HCC. The role of HSP27 in HCC metastasis warrants further investigation.

9.7 Role of the Carboxyl Terminus in the Regulation of Phosphatidylethanolamine N-methyltransferase (PEMT)

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Phosphatidylethanolamine N-methyltransferase (PEMT) is a membrane protein that catalyzes the methylation of phosphatidylethanolamine (PE) to phosphatidylcholine (PC). The expression of this enzyme has been found to be inversely correlated with hepatocyte carcinogenesis. Human PEMT enzyme is composed of 199AA, with both N- and C- termi exposed to the cytosolic surface where it can interact with S-adenosylmethionine (AdoMet). In this study, we tested the role of C-terminus in the enzyme regulation by transiently transfection of wildtype and truncated forms of PEMT into McArdle-RH7777 cells. When the last 6 amino acid closed to the C-terminus were deleted, neither the protein expression nor the enzyme activity was affected. However, both protein expression and activity were abolished when the last 13 amino acid were deleted. Real Time-PCR analysis revealed that the mRNAs from wildtype and truncated forms were successfully transcribed. These results suggest residues 187IYRQKAS193, within the C-terminus are important in maintaining the integrity and function of this membrane enzyme. In a subsequent study, we mutated Arg at residue 189 to Lys by site-directed mutagenesis and found this mutation lead to abolished enzyme activity. Our result indicates that Arg 189 is crucial for the PEMT activity. The mechanism by which the Arg 189 to Lys mutation diminished the enzyme’s activity could involve disruption of the interactions between the enzyme and its substrate.
9.8 A Combinational Strategy to Phosphoproteome of Mouse Liver

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Protein phosphorylation is a crucial and ubiquitous post-translational modification regulating activities of many proteins. Reversible protein phosphorylation plays an essential role in a variety of fundamental cellular functions. In the post-genomic era, many methods have been employed to study the phosphoproteome and each has its strengths and limits. Here we reported a combinational strategy, including phosphoprotein affinity capture, protein phosphatase treatment, immunoblotting (anti-pSer, anti-pThr, anti-pTyr), a newly developed fluorescence staining-Pro-Q Diamond and shotgun analysis, to unbiasedly decipher mouse liver phosphoproteome.

Proteins of C57 mouse liver tissue were divided into two aliquots, one of which was dephosphorylated using recombinant lambda protein phosphatase, and the other was not treated with the enzyme. The two aliquots were subjected to 2-D electrophoresis, then stained with Pro-Q Diamond and post-stained with Sypro Ruby, immunoblotting was performed as well. On the other hand, we enriched phosphoproteins by phosphoprotein purification system (QIAGEN) and eluted proteins were identified by linear ion trap mass spectrometry (LTQ, Thermo Electron Corporation) shotgun analysis.

We detected 61 differential protein spots (more than 2.0 fold) after PPase treatment, these spots will be identified by LTQ and their phosphorylation states will be confirmed by fluorescence staining or immunoblotting. Moreover, we identified 357 different proteins in shotgun analysis, and bioinformatics analysis indicated that phosphoproteins in mouse liver showed a diversity of subcellular location with 64% of them localized in cytoplasmic and nuclear, involved in almost all kinds of metabolic activities such as protein sorting, protein degradation, cell apoptosis and PKC pathway, which suggested that phosphoproteins play an important role in physiologic function performed by liver. Such efforts have paved the way for the comparative phosphoproteome analysis to favour in understanding the mechanism leading to pathological states such as hepatocellular carcinoma and other diseases.

9.9 Differential Secretome Analysis of Hepatocellular Carcinoma

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Hepatocellular carcinoma (HCC) is the most common primary hepatic tumor and one of the most common cancers worldwide. A number of HBV patients with chronic hepatitis will develop hepatocellular carcinoma. In order to find out the molecular markers for the diagnosis and prognosis of Hepatitis B Virus (HBV)-associated HCC, in this study we have used Isotope-Coded Affinity Tag (ICAT) to inspect the secretome difference expression between Hep3B and Chang liver cell lines.

Cleavable ICAT analysis showed that 181 proteins were differentially expressed in secretome of Hep3B and Chang liver with more than 2-fold difference. Using the secretome differential expression we found some proteins with higher expression in Hep3B than Chang liver, such as zinc-induced metallothionein 2A/1A, Metallothionein-1A, Metallothionein-II, MAP4K5 protein, protein tyrosine phosphatase, AMBP protein precursor, migration stimulation factor FN70. These different expression proteins will be further investigated for function elucidation.

9.10 Krüppel Zinc Finger (ZNF) Proteins in Human Fetal Liver

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Between 564 and 706 Krüppel ZNF genes presented in the human genome, thereby constituting one of the largest gene families in human. Krüppel ZNF proteins are important in the regulation of a number of diverse biological processes, such as cell growth, differentiation, embryogenesis and tumorigenesis. 1950 proteins are identified from human fetal liver in our laboratory, and among them 169 are Krüppel ZNF proteins with their coding genes existed on 17 chromosomes. Notably, chromosome 19 codes 101 Krüppel ZNF proteins. According to the number of ZNF motifs the proteins carried, Krüppel ZNF proteins are divided into two groups, the first group consists of proteins with five finger motifs or less, they seem to be involved in cellular housekeeping or in the regulation of development; the second group consists of proteins with more than five finger motifs, they probably evolved later and have only been found in vertebrates. Among the 169 Krüppel ZNF proteins, only 2 proteins are found belonging to the first group. KRAB-ZNF proteins are Krüppel-related ZNF proteins containing the KRAB domain presented in the proteins N-terminus. They are considered as regulators of hematopoiesis. We find 123 KRAB-ZNF proteins, the result strongly supports that fetal liver is a hematopoiesis-related organ.
9.11 Identification and Characterization of Phosphorylated Proteins in Human Fetal Liver by Using Immobilized Metal Affinity Chromatography and Tandem Mass Spectrometry

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Protein phosphorylation is one of the most important reversible post-translational modifications that occur in cells. Analysis of the entire phosphorylated proteins in cells, or the "phosphoproteome," has become a key part of functional proteomics recently.

Human fetal liver aged 16–24 wk of gestation corresponds to the turning point between immigration and emigration of the hematopoietic system. The phosphoproteome of human fetal liver will complement the knowledge of genome, transcriptome and proteome, and will be very meaningful to understand the important function of liver.

We report here the phosphoproteomics analysis of membrane proteins from human fetal liver based on the application of immobilized metal affinity chromatography (IMAC), capillary reverse phase HPLC and tandem mass spectrometry.

HFL membrane proteins were digested in solution and the resulted peptides were enriched by using IMAC followed reverse phase HPLC and tandem MS. The MS/MS data were searched against the protein sequence database with the search engine MASCOT. Based on the database searching and manual interpretation of MS/MS spectra, 28 phosphopeptide sequences representing 21 different HFL proteins and 36 phosphorylated sites were confirmed. Most of the phosphorylation occurred in serine residue (35 sites) and one phosphorylation occurred in tyrosine residue. About 27 identified phosphorylated sites were not previously reported to be phosphorylated.

In order to get more valuable information about the identified phosphorylated proteins, three prediction programs, Scansite, ScanProsite and NetPhos, were used to predict the possible phosphorylated sites and their kinase from the primary protein sequences. Of the total 36 phosphorylated sites defined by using tandem MS, only 3 sites were predicted by all three programs and were predicted by program Scansite and ScanProsite as the substrate of casein kinase II.

9.12 Functional Characterization of RhoC and Rab14 in Liver Cancer Cells

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Hepatocellular carcinoma (HCC) is one of the most common internal malignancies worldwide. In this study, two Ras-superfamily genes, RhoC and Rab14, which are small guanosine triphrophatases (GTPases), have been characterized. Members of the Rho family are involved in the regulation of various cellular processes, such as microfilament network organization, cell cycle control and malignant transformation. We compared RhoC and Rab14 mRNA and protein expression in normal human liver tissue and HepG2/Hep3B hepatoma cell lines and found that both RhoC and Rab14 showed significantly higher expression level in HepG2 and Hep3B hepatoma cells when compared with that of the corresponding normal liver sample. Studies with green fluorescent protein (GFP) fusion protein of RhoC and Rab14 suggested that they are located in the endoplasmic reticulum (ER). Cell cycle studies showed that RhoC may induce cell progression by inducing G1 to S phase transition while Rab14 may induce cell proliferation with an increase in DNA synthesis. Meanwhile, no apoptotic effect was found by DNA fragmentation analyses when RhoC or Rab14 was transfected into liver cells. Downstream signaling pathway induced by RhoC and Rab14 expression in liver cells has also been investigated in our study. Furthermore, transient transfection of RhoC in normal mouse hepatocytes and normal human hepatocytes induced stress fibers formation and triggered reorganization of actin cytoskeleton. Thus our results are consistent with the observation that RhoC is associated with highly invasive HCC and that the rearrangement of actin cytoskeleton is crucial for cancer invasion and metastasis.
9.13
Protein Expression Profiling—Targeted Proteomic Techniques for Quantifying Cytochrome P450 Enzymes

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The primary site of drug metabolism is the liver where drugs are transformed by metabolizing enzymes, in particular the cytochrome P450 enzyme system. This is a superfamily of heme proteins primarily responsible for phase 1 drug metabolism.

There are 4 major families of P450 isoforms in humans with ~25 different isoforms, each with different substrate specificities inducible by different drugs or chemicals. The changes in expression of the different P450 proteins provide information on the toxicity of different drugs.

A method for the absolute quantitation of cytochrome P450 proteins in liver using ICAT® reagent-labeled synthetic peptides and a hybrid quadrupole linear ion trap mass spectrometer was investigated.

Peptides specific to individual P450 isoforms were synthesized and labeled with the stable isotope tag (light Cleavable ICAT® Reagent). These peptides were used to create a standard concentration curve using quantitative Multiple Reaction Monitoring (MRM) scans. Mouse liver microsome samples (wild-type and drug induced) were then labeled with heavy cleavable ICAT reagents. The high sensitivity and specificity of the triple quadrupole-based MRM scan on the hybrid quadrupole linear ion trap system was used to both detect and quantify the specific P450 peptides in this complex liver microsome digest mixture. Comparison of the chromatographic areas of the heavy peptide in the sample to the standard concentration curve of the light peptide provided accurate quantitation of the peptide and therefore of that protein in each liver sample. To date, 14 different P450 proteins have been quantified in each sample, many showing changes in expression upon induction. In addition, because a concentration was determined for each protein, it was determined that the levels of expression of these p450 proteins varies greatly (>100 fold).

9.14
Proteomic Analysis of Hypoxia-associated Protein in HepG2


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Hypoxia has been suggested as a driving force in angiogenesis in cancers. To further investigate the role of hypoxia during cancer development, we used human hepatoma cells (HepG2) and analysed the protein expression profile change using 2-dimensional polyacrylamide gel electrophoresis (2DE) following hypoxic treatment. 19 protein spots whose expression levels showed significant changes under hypoxia stress were found. Of these spots, 18 were identified successfully with peptide mass fingerprinting (PMF) using matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) after tryptic in-gel digestion. These proteins exhibited various cellular functions, including 1) stress response pathways; 2) cytoskeletal structure; 3) signal transduction; 4) cell survival/Angiogenesis; 5) glycolysis and glucose uptake; 6) translation; 7) ion transport and metabolism and 8) amino acid transport and metabolism. Our results indicate that hypoxia induces cancer-associated proteins and might be an important therapeutic target.

9.15
Proteomic Signature Corresponding to Early Intrahepatic Recurrence of Hepatocellular Carcinoma

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Poor prognosis of hepatocellular carcinoma (HCC) is mainly attributable to a high incidence of intrahepatic recurrence after surgery. To develop the accurate predictive marker for early intrahepatic recurrence and to identify therapeutic targets for the treatment of HCC patients, we examined protein expression profiles in HCC tissues using two-dimensional difference gel electrophoresis (2D-DIGE) representing approximately 1500 protein spots. An initial sample set (a training set) was comprised of 27 HCC patients; 12 patients who had intrahepatic recurrence within 6 months after curative surgery and 15 patients who did not have recurrence within 2 years. Using a supervised classification method based on support vector machine algorithm, we generated for the first time a proteomic signature, comprising of 23 protein spots, which associated with the early recurrence. The predictive performance of the signature was validated by a test set comprising 13 newly enrolled HCC patients; 6 early recurrence and 7 non-recurrence cases. The signature correctly classified 27 HCC patients in the training set, and predicted early recurrence in 12 (82.3%) of 13 patients in the test set with a positive predict value of 100% and a negative predict value of 85.7%. Mass spectrometric protein identification revealed that the proteins corresponding to the spots have the properties of oxidoreductases, chaperone, annexin and so on. In conclusion, we identified the proteomic signature, which can be a predictive marker for early intrahepatic recurrence. The proteins in the signature can also be the therapeutic targets for HCC.
9.16 Protein Expression of the Rat Liver Exposed to TCDD
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Dioxins are a class of polyhalogenated aromatic hydrocarbons that induce a wide spectrum of toxic responses in experimental animals. In this study, two SD rat groups were exposed to 2,3,7,8-tetrachlorobenzop-dioxin (TCDD): one group with short-term exposure at a single dose of 1, 10, 20, and 50 µg/kg body weight (group 1) and the other with long-term exposure at daily-and-low dose of 0.01, 0.1, 1 and 2.5 µg/kg body weight (group 2) for a month. To resolve the protein profile of rat liver which was exposed to TCDD at different doses, two-dimensional electrophoresis (2-DE) was utilized. In group 1, as the result of 2-DE, two newly expressed spots and seven volume-increased spots were detected and identified by ESI-Q-TOF MS/MS; especially, proteasome subunit beta type 3 was increased in all doses. In addition, as the result of 2-DE for group 2, six volume-increased spots were screened. Particularly, histidine triad nucleotide binding protein was increased in both 0.1 and 1 µg/kg dose. The identified proteins were confirmed using Western blotting. Among the identified proteins, it appeared that apolipoprotein A-IV might protect lipid peroxidation induced by TCDD exposure.

9.17 LAPTM4B, a Novel Member in Transmembrane 4 Superfamily
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We cloned, characterized and studied the expression patterns of a novel member of TM4-SF, lysosme-associated protein transmembrane 4 beta(LAPTM4B). This gene was found by differential display of four types of human liver tissues, including normal adult liver (NL), fetal liver (FL), hepatocellular carcinoma (HCC) and paired noncancerous liver (PNL).

LAPTM4B is highly expressed in testis, muscle and heat, but low in NL. The expression is medially upregulated in FL, and highly upregulated in 87.3% of HCC. LAPTM4B encodes two proteins (LAPTM4B-35 and -24) with antagonistic functions. The LAPTM4B-35, but not LATM4B-24, is highly upexpressed in HCC, thus the ratio of LAPTM4B-35/LAPTM4B-24 was dramatically raised, whereas in PNL the ratio was kept at the same level as NL.

The upregulated ranges of LAPTM4B-35 at mRNA and protein levels were both inversely correlated with HCC differentiation. Here we address the role of LAPTM4B in carcinogenesis. The transfection experiments indicated that overexpression of LAPTM4B-35 strongly promoted cell transformation, including promotion of survival, proliferation, migration and invasion, and was tumorigenic; whereas LATM4B-24 inhibited colony formation and induced apoptosis. Also, the over-expression of LAPTM4B-35 activated c-myc, c-fos and c-jun, and upregulated cyclin D1 and cyclin E, suggesting that LAPTM4B might be a novel protooncogene. The study on signaling pathway demonstrated that LAPTM4B-35 might be an assembling platform of signaling molecules: the formation of the LAPTM4B-35—a6β1 integrin (the receptor of laminin); the formation of the LAPTM4B-35—a6β1 complex was enhanced by plating the cells onto laminin substrate, suggesting that the a6β1 receptor and EGFR may be integrated within plasma membrane via LAPTM4B-35. We also evidenced that LAPTM4B-35 interacted with PI3K and PKCδ, and activated FAK, MAPK and AKT, indicating that LAPTM4B-35 is involved in FAK/MAPK and PI3K/AKT signaling pathways. There are several phosphorylation, glycosylation and liposylation sites in LAPTM4B molecules. The relationship between modifications of LAPTM4B protein and functions is being studied. Now we know that the Tyr285 of LAPTM4B was peakfully phosphorylated when the HCC cells were plated on to laminin substrate; and the phosphorylation was totally inhibited by the antibody direct the EC2 domain of LAPTM4B.