8.1

Identification of Hepatocellular Carcinoma-associated Serum Glycoproteins by Lectin Affinity Purification and 2D Gel Analysis

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Identication of early hepatocellular carcinoma (HCC) in patients with chronic liver diseases (CLD) is an important clinical task. Previous studies have shown that patterns of alpha-2,6-sialylation and alpha-1,6-fucosylation change in liver cancer, leading to aberrant glycosylations on some serum proteins. In this study, we purified alpha-2,6-sialylated and alpha-1,6-fucosylated serum glycoproteins by using lectin SNA- and LCA-affinity chromatography, repectively. The purified serum glycoproteins from 10 CLD patients and 20 HCC patients were then separated by two-dimensional polyacrylamide gel electrophoresis, and the resulted gel images were compared. When analyzing alpha-2,6-sialylated proteins, 85 spots were differentially expressed between HCC and CLD groups, corresponding to 43 glycoproteins ($p < 0.05$). When analyzing alpha-1,6-fucosylated proteins, 27 spots were significant different between the two groups, corresponding to 12 glycoproteins ($p < 0.05$). The protein identities of the differential glycoproteins were uncovered with MALDI-TOF-TOF MS. Haptogloblin was identified to be up-regulated in HCC in both sialylation and fucosylation analyses. By immunoassay, increased serum haptoglobin level was confirmed in independent cases comprising 40 HCC and 30 CLD patients. This is the first study showing the increased serum haptoglobin levels in the HCC patients. Serum haptoglobin, particularly its fucosylated variants, is a potential tumor marker of HCC. (The study was supported by the RGC Earmarked Grant 4466/03M from the University Grants Committee of Hong Kong.)

8.2

Comparative Proteomic Analysis of Rat Liver Fibrogenic Cells of Different Origins

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Liver fibrosis is the consequence of abnormal extra cellular matrix deposit which has been ascribed to myofibroblastic cells. Myofibroblasts are commonly considered to derive from hepatic stellate cells (HSC) known to play a major role in vitamin A storage. Following liver injuries, HSC undergo activation and acquire myofibroblastic features. Other cell types may be involved in the fibrogenic process; in particular, periductal fibroblastic cells could be able to transdifferentiate into peribiliary myofibroblasts in biliary-type liver fibrosis. So far, specific markers of the cell origin(s) at the stage of myofibroblasts are lacking. We investigate a two-dimensional electrophoresis analysis for myofibroblasts deriving from HSC and peribiliary myofibroblasts. Their proteome comparison could lead to find new cell type specific markers and may help to better understand the contribution of the different fibrogenic cell types in liver fibrogenesis.

HSC and periductal fibroblastic cells were isolated from healthy Sprague-Dawley rat livers. Cells were cultured until passage two as they present a myofibroblastic phenotype. Isoelectrofocusing was realized with IPG gels (4–7, 5,5–6,7) and 10% SDS-PAGE. Proteins were visualized by silver staining. The proteome patterns on 2D-gels of HSC myofibroblasts and peribiliary myofibroblasts were very similar suggesting that these two cell types exhibit little phenotypic difference at the stage of fully differentiated myofibroblasts. Yet, five proteins with different expression patterns were readily detected in three separate gels issued from three different culture preparations. These proteins are currently identified, using MALDI-TOF and LC-MS/MS.

8.3

Proteomic Roadmap of Mouse Liver Development from Embryo to Adult

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Liver is a major metabolic organ in adult, whereas it also plays critical roles in hematopoietic organogenesis in mammalian embryos. There is a great interest to identify liver proteins that are associated with different developmental stages. However, global protein expression analysis of liver development from embryo to adult remains largely unknown. This study aims to detect which proteins are likely to be involved in the process of liver development and differentiation by 2-DE protein expression profiling. Proteins that show consistent significant variations in expression quantity during the process are scrutinized, and the functionality of the proteins of interest revealed by clustering. Liver organs obtained from BALB/c mice (n = "5," each group) at E13.5, E16.5, 0 dpp, 21 dpp and adult, were subjected to 2-DE gel electrophoresis. Each experiment was repeated at least twice. A total of 396 protein spots showed statistically significant variations between the five different groups by one-way ANOVA. Each stage of liver development was correctly classified by distinct proteomic patterns using unsupervised computer algorithms. Furthermore, clustering analysis revealed five distinct functional groups of proteins which appear: (i) only in embryonic stages, but disappeared before birth; (ii) only in embryonic stages, but disappeared after birth; (iii) in a narrow window during birth; (iv) during birth and through adulthood, but absent in embryo; and (v) after birth and in adult liver. Identification and characterization of these differentiating protein species will shed light on the proteomic roadmap of liver development from embryo to adult stages.

8.4

Age Peculiarity in Regulation of Liver Aldehyde Reductase Activity by Free Radical Oxidation Products

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Aldehyde reductase plays an important part in cell adaptation to oxidative stress. It can be assumed that aldehyde reductase activity is regulated by the products of free radical oxidation and reactive oxygen species. This mechanism of enzyme regulation may be connected with forming resistance to oxidative stress injury and can change in the course of ontogenesis. All that considered our investigation was designed to assay age-related effect of hydrogen peroxide and propional on aldehyde reductase activity in adult and old rats liver. The data obtained demonstrated that hydrogen peroxide and propional, which had accumulated during oxidative stress, took part in the regulation of aldehyde reductase activity. Incubation of liver post mitochondrial fraction with 10 mmol propional was shown to cause a decrease in aldehyde reductase activity. The liver enzyme from old rats demonstrated maximal sensitivity to aldehyde inhibition by aldehyde. Hydrogen peroxide (10 mmol) brought about aldehyde reductase activation in liver of the adult rats and to its inhibition in the old ones. Age-related deviations in the effect of hydrogen peroxide and propional on the liver aldehyde reductase activity can be associated with age-related differences in the state of enzyme polypeptide chains and with disfunctions in adaptive reaction restricting accumulation of hydrogen peroxide and endogenous aldehydes in hepatocytes. Age peculiarities in manifestation of regulatory effect of those metabolites on aldehyde reductase were accompanied with an increase in the enzyme activity in the liver of adult rats and its decrease in the liver of old animals. The changes observed can be important in reducing hepatocyte resistance to oxidative stress injury in ageing.

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8.5

Mapping of Human Liver Proteins by DIGE

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Non-alcoholic fatty liver disease includes a spectrum of alterations that range from the accumulation of triglycerides in the hepatocytes (steatosis) to steatosis with hepatic inflammation (steatohepatitis or NASH). NASH, in turn, progresses to cirrhosis and hepatocellular carcinoma (HCC). The mechanisms underlying the manifestation of NASH are unclear, but it is associated to obesity, insulin resistance and diabetes. Recent studies in our laboratory showed that S-adenosylmethionine (AdoMet) helps maintain normal liver function, since chronic hepatic deficiency results in the spontaneous development of NASH and HCC. In previous work, our group used knockout mice deficient in hepatic AdoMet synthesis (MAT1A-KO) to carry out a functional proteomics study. This mouse model spontaneously develops oxidative stress, NASH and HCC. Out of 117 identified proteins whose levels are altered when compared to wild-type mice, only 12 maintained the altered expression level to the onset of histological lessions. Some of these differentially expressed proteins were confirmed in human samples. The aim of our present project is to establish a map of human liver proteins to use as a reference for the study of liver samples from NASH and HCC patients. To this end, we have gathered liver samples from 10 healthy individuals and we are working on the proteomic separation of the proteins using two-dimensional difference gel electrophoresis (DIGE), using a pooled internal standard made from the combination of all samples. This technique allows us to determine the range of variation of the position and intensity of protein spots from one sample to the other, taking advantage of the presence of the internal standard, which minimizes the gel-to-gel variation. The patterns of variability in protein expression levels among healthy individuals will be used as a basis for the identification of alterations in protein expression in NASH and HCC patients. Protein identification will be carried out by MALDI-TOF/TOF mass spectrometry.

8.6

Generation and Characterization of Hybridoma Cell Lines against Human Liver Cytosolic Proteins by Using Fractionated Proteins as Immunogens

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Human liver plays crucial functions in metabolism of biological large molecules, energy production, detoxification, and immune responses. Analyzing liver proteome is the essential approaches for elucidating liver functions, as well as new drug targets discovery for liver diseases. In this point, large amount of well-characterized antibodies against native proteins for protein expression, quantification, localization, and modification as well as protein-protein interactions, are needed. To fulfill this emerging need for large quantities of antibodies, we generated a monoclonal antibody (mAb) bank against human liver cytosolic proteins. Normal adult liver tissues were homogenized and subjected for fractionation by gel filtration and ion exchange chromatography, and 16 fractions were obtained to immunize BALB/c mice for generating mAbs. To identify the antigen specificities of each mAb, we used our established technical platform, including ELISA, immunoprecipitation followed by MALDI-TOF MS, and screening of cDNA expression library. Totally, about 300 mAbs were established against 14 fractions of liver cytosolic proteins when the hybridomas were screened with respective fractions. Most of these mAbs (228/236) were tested with 13 liver abundant proteins by ELISA. We found that 24 of 236 mAbs (10%) had single specificity against 9 proteins, 124 (52%) mAbs had multi-specificities, 79 (33%) mAbs did not recognize any of the liver abundant abundant proteins. All of the mAbs were subjected to Western blot analysis under both reduced and non-reduced conditions. 158 (67%) mAbs gave specific signals in Western blotting when using reduced SDS-PAGE, while 153 (65%) mAbs were positive for non-reduced samples. Among 36 mAbs, which subjected to MALDI-TOF analysis after immunoprecipitation by selected mAbs, 13 protein antigens were identified. Some mAbs' specificities (53) were further analyzed by screening of human liver cDNA expression library and 9 specific proteins recognized by 24 mAbs were identified. These identified protein antigens were immunoglobulin gamma 1, ferritin light polypeptide, aldehyde dehydrogenase, glutathione transferase omega 1, glyoxylate reductase/hydroxypyruvate reductase, fibronectin, serum albumin precursor, elongation factor 2, and alcohol dehydrogenase alpha chain. Overall, our data indicated that the strategy of using fractionated proteins to generated mAbs against human liver cytosolic proteins is one of the options for large scale preparation of mAbs especially for native proteins.

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8.7

Subcellular Preparation and Evaluation of Mouse Liver

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Organellar proteome has become one of the most important field of proteomics and the subcellular fractionation and purification of organelles had always been a challenge for cell biologists. The liver tissues of the C57 mouse are used to find the optimum method of subcellular preparation for the HLPP preview of subcellular fractionation. We select the method to get the multiple fractions from a single tissue, which can take full advantage of the limited sample. The protocol yields five fractions: plasma membrane, mitochondria, nucleus, endoplasmic reticulum and cytoplasm. The subcellular specificity, purity, integrality and enrichment fold were demonstrated by immunoblot analysis of organelle marker proteins, transmission electron microscope and enzymatic analysis. The selected organelle marker proteins include COX IV, Cytochrome c and VDAC for mitochondria, Lamin B for nucleus, KDEL for endoplasmic reticulum, Flotillin-1 and Caveolin for plasma membrane and aldehyde reductase for cytoplasm. 3 kinds of sample preparation methods for subcellular fractionation are evaluated: fresh tissues, frozen homogenized tissues and frozen tissues. Immunoblot analysis, transmission electron microscope and protein yield support that the fresh tissues superior to the frozen homogenized tissues, and the frozen homogenized tissues superior to frozen tissues for subcellular preparation. Taken together, the recommended subcellular preparation method of HLPP is the fresh sample, the frozen homogenized tissues is possible if the lab can not get the fresh samples timely and the frozen tissues is not recommend.

8.8

Comparative Analysis of 2D Blue Native Gel Profiles of Rat Liver Mitochondria Proteins during Liver Regeneration

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Mitochondria play important roles in liver regeneration. To explore the mitochondrial role in the process, we studied the protein expression levels in mitochondria during liver regeneration and identified the differentially expressed proteins. Two dimensional blue native gel electrophoresis (2D BN/PAGE), which is a powerful tool to study hydrophobic protein complexes besides hydrophilic proteins, was used to separate mitochondrial proteins. To this purpose, highly purified mitochondria were isolated with strictly quality control system, which includes electron microscope for mitochondrial anatomic integrate evaluation and immuno-blotting assay for contamination from other organelles. The mitochondria proteins were resolved by 2D BN/PAGE and the profiles produced by 2D BN/PAGE were analyzed by PDQuest with two fold significance threshold. Further analysis by ESI-Q-TOF revealed 4 up regulated proteins and 16 down regulated proteins in 24 hours regenerated liver tissue compared to control, respectively. The up-regulated proteins include the proteins that are involved in fatty acid metabolism and acetyl-CoA metabolism. 22% of down regulated proteins are oxidative phosphorylation (OxPhos) complex subunits and the rest are involved in amino acid metabolism, Keton body metabolism and also proteins without GO function information. Our preliminary results indicate that not only OxPhos complexes are involved in the regulation of mitochondrial activity in the process of liver regeneration, but also the enzymes from mitochondrial matrix play essential roles.

8.9

Protein Expression Profile for Differentiating among Hepatocellular Carcinoma Cell Lines, Tumor Tissues, Adjacent Non-tumor Liver, and Normal Liver

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With an aim to identify proteins abnormally regulated in hepatocellular carcinoma (HCC), we have used a two-dimensional (2D) gel electrophoresis approach to profile and compare the global protein expression profiles of 14 hepatitis B virus-related HCC tissues, 14 adjacent non-tumor liver tissues, 10 normal liver tissues and 10 HCC cell lines. 2D gel protein spots were indexed and matched among the samples. Comparing the global expression profiles composed of 1913 protein spots between the paired tumor and non-tumor tissues, normalized intensities of 110 protein spots were significantly different (SAM, paired analysis, false discovery rate = 0.05). These 110 differential proteomic features were validated by comparing their quantities between tumor and normal liver tissues (SAM, unpaired analysis, false discovery rate = 0.05). Compared to normal liver, 35 and 56 of them were significantly up-regulated and down-regulated in HCC. Hierarchical clustering using the normalized quantities of these 91 differential proteomic features yield two major groups, one of which contained 100% of the HCC tissues and 100% of the HCC cell lines whereas the other contained 100% of the adjacent non-tumor liver tissues and 100% of the normal liver tissues. The HCC tissues were well discriminated from the HCC cell lines at 100% accuracy. The non-tumor liver tissues were discriminated from the normal liver tissues at 71% accuracy. The identities of these differential proteomic features were obtained with MALDI-TOF-TOF MS. According to their functions, they could be classified into various groups, including heat shock protein, chaperone, kinase substrate, cell signaling, apoptosis regulation, transcription regulation, free-radical scavenger and metabolic enzyme. Some of them may play important roles in cellular proliferation and oncogenic transformation of HCC. In conclusion, proteins differentially expressed in HCC were identified, and our results provide insights in the pathogenesis of hepatitis B virus-related HCC. (This study was supported by the Central Allocation Grant CUHK 2/02C from the University Grants Committee of Hong Kong.)

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8.10

Complexity Reduction Techniques in Liver Proteomics

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Tissues are complex structures consisting of compartments such as several types of cells and intercellular matrix. Cells themselves comprise nuclei, cytoplasm and intracellular organelles. This complexity contributes to the difficulties of interpretation of the results of proteomic studies. Isolation of individual tissue or sub-cellular compartments prior to proteome analysis greatly facilitates protein identification and interpretation of the results. To reduce the tissue complexity in liver proteome studies we employed laser capture microdissection (LCM), immunobead cell sorting and subcellular fractionation.

LCM from the cryostat sections of human liver biopsies was used to dissect cholangiocarcinoma cells from the surrounding tissue. The 2D electrophoresis of the dissected samples revealed several minor protein spots not detected prior to LCM. The proteome analysis of the dissected tumor compartment in comparison with normal liver tissue disclosed over 100 differentially expressed proteins, while twice as little differences were seen in the non-dissected samples.

Proteome studies of different cell types isolated from livers of 11, 17 and 26 week old fetuses aborted for medical reasons and from the biopsies of adult liver revealed gradual decline of the numbers of juvenile hematopoetic cells and increase of cells expressing hepatocyte proteins. Proteome analysis of liver tissue samples failed to demonstrate that clearly enough. We also analysed the proteome of the microsomes isolated from human fetal liver. These data are a good reference point for the post-translational genomics studies of human liver microsomes, because before birth no microsomal enzymes are induced by food ingredients and orally taken drugs (enzyme induction by substances in maternal blood may take place). Isolation of the microsomes allowed detection of some low copy proteins not spotted using whole liver tissue extracts.

8.11

3-D Schemes for Establishing the Proteome Reference Map of Human Liver

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To profile a tissue proteome with high complexity, traditional techniques such 2-DE-MALDI-TOF/TOF or 2-D shotgun analysis can only provide limited resolution. The pre-fractionation of proteins by the combination of different separation principles and the introducing of gas-phase fractionation (GPF) to facilitate peptide capturing are preferred in recent years.

To characterize the human liver proteome, two optimized 3-D schemes were adopted in this study. In the first one, SDS-PAGE gels with different concentration were used to resolve intact proteins. Tryptic peptides extracted after in-gel digestion were separated and analyzed by nano-LC-ESI-MS with GPF scan. In the second scheme, an intact protein separation was accomplished by RPLC with a high recovery rate preparative column and SDS-PAGE. Following with nano-LC-ESI-MS determination of the peptide extracted.

Both of the 3-D schemes were proved very effective in term of proteome profiling. After data was filtered by using DTAselect, more than 3000 liver proteins were determined in each of the schemes. Under the GPF mode, more peptides could be captured and resulted in the increasing detection of proteins by about 50 percent. Furthermore, the percentage of proteins that matched more than two peptides increased when GFP scan was performed, which indicate an increase in the reliability of the protein dataset. Using a RP prefractionation before SDS-PAGE, a similar productivity was obtained. The results of this study not only contribute to the establishment of a comprehensive reference map of human liver proteome, but also shed light on the selection of a proper combination of multiple separation techniques in proteome research.

8.12

Detection and Identification of New Tumor Markers in Human Hepatocellular Carcinomas

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The proteomic approach provides an excellent tool to detect and to identify proteins that are associated with cancer. In previous investigations on experimentally induced rat hepatomas we detected and sequenced a novel aldose reductase-like protein (rARLP) and characterized this member of the aldo-keto-reductase superfamily as a highly significant marker protein to be typical for rat hepatomas induced by genotoxic nitrosocompounds. The present study was intended to look for the presence of similar tumor-associated marker proteins in human hepatocellular carcinomas (HCC) which offer essential precondition for tumor-diagnosis and -therapy.

Comparative proteome analyses were performed with normal liver tissue and primary human HCC. New tumor-associated protein variants were detected by 2-DE and identified by MALDI-TOF-MS. Several new tumor-associated protein variants represent members of the aldo-keto-reductase superfamily. The human aldose reductase-like protein-1 (hARLP-1) was the most prominent tumor-associated variant. The enzyme was found as various distinct protein-species located at different M_r and pI in the majority of human HCC. In Northern blots only one hARL- mRNA was found, suggesting that the different hARLP-spezies are due to posttranslational modifications. A so far unknown tumor-associated protein, hARLP-5 was identified, different from hARLP-1 by one amino acid (D313N), as confirmed by cDNA sequencing, indicating two allelic forms of the human aldose reductase-like gene. A novel antibody directed against hARLP was able to detect all these single protein-spezies and revealed hARLP positivity in formalin-fixed, paraffin-embedded human HCC by immunohistochemistry.

Of all investigated human HCC samples 95% were positive for hARLP as proven by 2-DE analyses, immunoblotting and/or immunohistochemistry by the use of the novel antibody directed against hARLP. This suggests that this tumor-associated protein variant might represent a strong immunohistochemical diagnostic marker of human HCC.

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