Editorial Commentary

Proteomic profiling in liver cancer: another new page

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Provenance: This is an Invited Editorial article commissioned by Section Editor Dr. Rui Liao (Department of Hepatobiliary Surgery, The First Affiliated Hospital of Chongqing Medical University, Chongqing, China).

Comment on: Jiang Y, Sun A, Zhao Y, *et al.* Proteomics identifies new therapeutic targets of early-stage hepatocellular carcinoma. Nature 2019;567:257-61.

Received: 27 May 2019; Accepted: 03 June 2019; Published: 21 June 2019.

doi: 10.21037/tgh.2019.06.03

View this article at: http://dx.doi.org/10.21037/tgh.2019.06.03

Hepatocellular carcinoma (HCC) is one of the top lethal malignancies worldwide. HCC development is usually asymptomatic and diagnosed at the advanced stage of the disease, at which effective therapeutic options are much limited (1). Multi-kinase inhibitors Sorafenib and Lenvatinib are the two first-line drugs approved by the Food and Drug Administration (FDA) of USA for advanced HCC patients (2,3). However, the overall response rates and the survival benefits in patients treated with these agents are still far from perfect. More recently, the immunotherapy using the Nivolumab, the anti-PD1 antibody, has gained an accelerated approval by the FDA after a Phase II clinical trial as a second line therapy for advanced HCC patients (4). Although it is extremely encouraging that a subset of HCC patients showed complete cure after anti-PD1 treatment, however, the overall response rate is around 14.3%. Also, subgroup analysis has suggested that anti-PD1 therapy may be less effective in HCC with certain etiological background such as HBV infection, in which the host may be suffering from immune exhaustion which greatly limits the outcome of the therapy.

Lack of well-defined druggable targets limits the potential of personalized treatment for HCC patients

HCC is known to be highly heterogeneous. There is a high level of inter-tumoral heterogeneity among different HCCs and this has created a problem in effective treatment, particularly when most of the molecularly targeted drug treatments are given on a 'one-size-fits-all' basis without considering their genetic background (5). This is unlike the target therapies employed in other cancers in which patients are pre-stratified based on the presence of corresponding driver mutations. Stratification of patients with HCC is needed particularly for a personalized treatment. Much effort has been paid to stratify HCCs molecularly hoping to find ways to inform treatments for HCC as well as the outcome of patients. With the technological advancements in sequencing and protein profiling techniques, it is foreseeable that this situation will change in the near future.

Next-generation sequencing meets HCC and its limitation

HCC enters the era of next-generation sequencing in 2011 when the result of the first whole-genome sequencing analysis in a single case of hepatitis C virus (HCV)-related HCC sample was reported (6). After this, a significant number of independent sequencing studies was carried out by different research groups worldwide on HCC samples with different etiological backgrounds. The sequencing results have, on one hand, confirmed and strengthened some of our previously accepted viewpoints on the molecular carcinogenesis of HCC, and, on the other, have also provided us with plentiful novel insights and clarified our understanding on the gene mutations, viral-host genome interactions, epigenetic modifications and global transcriptomic changes in HCC (7-10). Although powerful and informative, genomic and transcriptomic studies have their limitations. For instance, changes at genomic and transcriptomic levels may not necessarily be translated

into protein levels and coupled with phenotypic changes. Also, post-translational modifications such as protein phosphorylation critical in regulating protein activity are usually missed and could not be faithfully represented solely by genomic profiling studies.

The early days of proteomics study in HCC

Since early this century, scientists have already envisaged capturing the underlying molecular changes not only at the DNA level but also at the protein level in HCC (11). Comparing to the global DNA profiling, global protein expression profiling is technically much more challenging due to the stringent requirements of high quality of tissues, as well as the proper extraction and detection of the cellular proteins of different abundance as much as possible (12). In the earlier days of proteomics studies in HCC, numerous small-scale proteomic studies were carried out in limited numbers of paired HCC samples, HCC-related cell lines or liver tissues from HCC-related transgenic animal models (13-15). With these approaches, some potential protein targets related to HCC metastasis, drug response or specific genetic alterations in HCC were identified. More importantly, the increased availability and progressive improvement in proteomics analyses have also stimulated the exploration to identify novel circulating HCC-related proteins which could potentially serve as more sensitive, alternative serum markers for early HCC diagnosis (16). Though most of the studies were carefully designed and executed, most of them suffered from limited sample size and low global protein coverage. Also, protein candidates identified in these studies were sometimes not validated in independent patient cohorts and lacked dedicated follow-up functional characterization, which greatly limits our understanding regarding their actual clinical significance and translational potential. With the significant advances of high-throughput protein analysis techniques, proteomics has offered a remarkably useful and versatile analytical platform for biomedical research. In recent years, different proteomic strategies have been widely applied in the various aspects of HCC studies, ranging from screening the early diagnostic and prognostic biomarkers to an in-depth investigation of the underlying molecular mechanisms.

The proteomics analysis in HCC: another new page

In a recent article published in Nature led by the Chinese Human Proteome Project Consortium, Jiang and her colleagues performed a large-scale proteomic and phospho-proteomic profiling in early-stage HCCs that were associated with chronic hepatitis B virus (HBV) infection (17). As a whole, over a hundred pairs of HCC and non-tumorous tissues were recruited and subjected to label-free, mass-spectrometry-based global proteomics analysis. They used quantitative proteomics analysis from the early-stage HCCs to stratify the cohorts into subtypes S-I, S-II, and S-III. S-III tumors had a more aggressive tumor behavior and more frequently had upregulation of proteins associated with oncogenic pathways such as TGF-β, HIF-1, integrin, and Rho GTPase pathways. Patients with S-III tumors also had the poorest outcome after surgery.

Furthermore, the authors found that patients with HCC subtype S-III exhibited a higher α-fetoprotein (AFP) level and more frequent microscopic vascular invasion in comparison with the other subtypes. In addition, and interestingly, the S-III subtype HCCs had more immune infiltration, with specific enrichment in M2-macrophages and immunosuppressive regulatory T cells, suggesting an immunosuppressive tumor microenvironment with T-cell exhaustion. These tumors also displayed proteomic markers of metabolic dysregulations, especially of glycolysis and cholesterol metabolism. Of significance, they found that sterol O-acyltransferase 1 (SOAT1) had high expression in the S-III subtype. SOAT catalyzes the formation of fatty acid-cholesterol esters.

The global protein coverage in this analysis was remarkable and, on average, over 5,000 proteins were identified in each tumor and non-tumorous liver samples. Interestingly, HCC tumors were found to express 20% more proteins when compared with the non-tumorous liver tissues, underlying a global increase of the corresponding mRNA transcripts. Also, the more aggressive the HCC tumors, as indicated by the presence of macroscopic invasion and higher AFP level, the more proteins were expressed. These findings actually highlight the critical changes in the global proteomic expression along with the tumor progression.

Patient stratification based on proteomic subtypes

Besides the global proteomic assessment, another outstanding feature of this study is the inclusion of phospho-proteomic analysis. Post-translation modification has long been recognized to act as an extra layer of

mechanistic control to regulate the biological activities of proteins. Protein phosphorylation is significantly involved in the signal transduction control, which in turn regulates numerous biological processes such as cell cycle control, balance between cell survival and cell death, and many other critical cellular functions. In human HCCs, hyper-phosphorylations have been detected in proteins involved in cell adhesion, cell proliferation, and transcription regulation. This observation further supports the notion that dysregulation of phosphorylation is another mechanism utilized by HCC cells to acquire their cancer-associated properties, but these changes could not be reflected by classical genomic, transcriptomic and proteomic analyses. More interestingly, in the current study, a subset of HCC patient samples was also subjected to whole genome sequencing for gene mutation profiling. The availability of the mutation landscape and phospho-proteome profiles in the same group of patients enabled cross-comparison and delineation of the dynamics between some previously identified HCC driver mutations and the downstream pathway activities. For example, a significant increase in S6 protein phosphorylation due to mTOR kinase activation was observed and confirmed in HCC patients carrying the Tuberous Sclerosis Complex (TSC) mutation (18).

To establish the prognostic significance of the global proteomic profiling data in HCC, they used a technique called non-negative matrix factorization consensus clustering (NMF) in this study to stratify the HCCs into the different proteomic subtypes, S-I, -II and -III. Unlike S-I tumors, which primarily showed upregulation of liver metabolic protein expression, S-II and III tumors tended to express more proteins involved in proliferative functions. In addition, S-III tumors further showed an increase in proteins supporting signaling pathways contributing to the aggressive tumor characteristics as well as the metabolic reprogramming in glycolysis and cholesterol metabolism. More importantly, S-III tumors were characterized by the gain of an immunosuppressive tumor microenvironment through a significant increase in the immunosuppressive marker expression and the presence of the immunosuppressive cell infiltration including M2 macrophages and regulatory T cells into the liver.

SOAT1 as a potential therapeutic target in proteomic subtype III HCC

Given that S-III patients have a poor prognosis, Jiang *et al.* further evaluated and examined the therapeutic value of targeting critical protein elevated in these patients. SOAT1, a protein target which was associated with the highest risk score for mortality was specifically followed up. Also known as ACAT1 (acetyl-CoA acetyltransferase), SOAT1 (Sterol O-acyltransferase 1) is one of the two enzymes responsible in catalyzing the synthesis of the cholesterol esters by joining the fatty acyl-CoA to the free cholesterol molecules. Cholesterol esters are important cholesterol derivatives, which play a critical role in cellular cholesterol storage as well as cholesterol transport in the bloodstream. Interestingly, SOAT1 activation has also been implicated in other pathological conditions such as Alzheimer disease (AD) development. Knocking out of SOAT1 or SOAT inhibition could attenuate the accumulation of amyloid β (Aβ) peptide, which may serve as a potential therapeutic strategy for AD (19). Dysregulation of SOAT1 has been implicated in human cancer and its expression was found to be upregulated in brain, prostate and pancreatic cancers (20-22).

In the study by Jiang *et al.*, SOAT1 was significantly upregulated in the HCC tumors and this upregulation was coupled with the upregulation of cholesterol esters, its catalytic product. Consistently, upregulation of SOAT1 protein was associated with poorer prognosis in multiple HCC cohorts and was shown to be an independent prognostic marker. Functionally, shRNA mediated SOAT1 knockdown could suppress cell proliferation and cell migratory ability in HCC cell lines. More importantly, the therapeutic potential of targeting SOAT1 was demonstrated by the ability of SOAT1 inhibitor, Avasimibe, in suppressing the growth of multiple patient-derived HCC tumor xenografts with high SOAT1 expression. By performing a proteomic comparison between the SOAT1 suppressed and the control cells, the authors proposed a model explaining that SOAT1 is critical in maintaining the plasma membrane cholesterol level, which is ultimately required for the proper localization of various transmembrane receptors in supporting HCC cell growth and metastatic related functions. Besides SOAT1, Jiang *et al.* further demonstrated that other enzymes playing different roles in the cholesterol homeostasis were significantly upregulated in human HCCs, suggesting that dysregulation cholesterol homeostasis may also play an oncogenic role in aggressive HBV-related HCC, and is not limited to non-alcoholic steatohepatitis (NASH)-associated HCC (23). Recently, the role of cholesterol biosynthesis pathway in supporting HCC development has been further demonstrated by two independent studies. Moon *et al.* have demonstrated that loss of the tumor suppressor p53 could activate the mevalonate

pathway in driving the HCC development by promoting the maturation of the sterol regulatory element-binding protein 2 (SREBP2), the master transcription regulator of the cholesterol synthesis pathway (24). Conversely, blocking the cholesterol biosynthesis gene was sufficient to block the liver tumor formation driven by a p53 loss in mouse. Additionally, Che *et al.* have demonstrated that liver cells without fatty acid synthase (FASN) could alternatively utilize the cholesterol synthesis pathway to support c-MET oncogene-mediated liver tumor formation through the upregulation of SREBP2 (25). Taken together, these studies provide additional evidence that cholesterol biosynthesis pathway is indeed required for HCC development.

Future perspective

The study by Jiang *et al.* has comprehensively demonstrated that the proteomics analysis can serve as a powerful tool in uncovering previously unidentified protein targets in HCC with encouraging therapeutic potential. Besides SOAT1, the current study has also highlighted a number of potential targets associated with different proteomic subtypes which are attractive targets to be functionally validated. The current study marks the beginning of a new chapter of the molecular characterization and classification of HCC, and we look forward to more proteomic studies in the future which will provide us with opportunities to further understand HCC tumors with other etiological backgrounds. As patients would greatly benefit from early detection of HCC, the complementary study of HCC-associated proteins in serum samples using state-of-the-art proteomics would also be a very attractive direction to be explored in the future.

Acknowledgments

Funding: This work was supported by Hong Kong Research Grants Council Theme-based Research Scheme (T12-704116-R) to IOL Ng.

Footnote

Conflicts of Interest: IOL Ng is Loke Yew Professor in Pathology. LK Chan has no conflicts of interest to declare.

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doi: 10.21037/tgh.2019.06.03
Cite this article as: Chan LK, Ng IO. Proteomic profiling in liver cancer: another new page. Transl Gastroenterol Hepatol 2019;4:47.

