Large-Scale Proteome Quantification of Hepatocellular Carcinoma Tissues by a Three-Dimensional Liquid Chromatography Strategy Integrated with Sample Preparation
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Supporting Information

ABSTRACT: Hepatocellular carcinoma is one of the most fatal cancers worldwide. In this study, a reversed-phase−strong cation exchange−reversed-phase three-dimensional liquid chromatography strategy was established and coupled with mass spectrometry to investigate the differential proteome expression of HCC and normal liver tissues. In total, 2759 proteins were reliably quantified, of which, 648 proteins were dysregulated more than 3-fold in HCC liver tissues. Some important proteins that relate to HCC pathology were significantly dysregulated, such as NAT2 and AKR1B10. Furthermore, 2307 phosphorylation sites from 1264 phosphoproteins were obtained in our previous phosphoproteome quantification, and the non-phosphorylated counterparts of 445 phosphoproteins with 983 phosphorylation sites were reliably quantified in this work. It was observed that 337 (34%) phosphorylation sites exhibit significantly different expression trends from that of their corresponding nonphosphoproteins. Some novel phosphorylation sites with important biological functions in the progression of HCC were reliably quantified, such as the significant downregulation of pT185 for ERK2 and pY204 for ERK1.

KEYWORDS: Human liver, hepatocellular carcinoma, proteome and phosphoproteome quantification, three-dimensional liquid chromatography separation

INTRODUCTION
Hepatocellular carcinoma (HCC) is the most common primary hepatic tumor and is one of the most frequently occurring cancers worldwide. Newly developed technologies in genomics, proteomics, and metabolomics have been applied to investigate the molecular mechanisms of tumor initiation and progression of HCC. Great technological progress has been made in the field of high-resolution mass spectrometry (MS)-based proteomics in recent years, and thousands of proteins can be investigated in one experiment for complex biological samples, such as tissues and cell lines. Comparative proteome quantification of the HCC and normal liver tissues is essential to elucidate the mechanisms underlying the occurrence and progression of HCC and is helpful in the discovery of new candidates for early HCC diagnosis. Stable isotope labeling strategies are widely utilized for proteome quantification due to their high quantification accuracy. Two types of stable isotope labeling strategies have been developed in recent years. The first strategy is in vivo metabolic isotope labeling, such as stable isotope labeling with amino acids in cell cultures (SILAC), which introduces isotopes at the protein level by using isotopic lysine (K) and arginine (R) during cell culture. The other strategy is in vitro chemical isotope labeling, such as isobaric tag for relative and absolute quantification (iTRAQ) and dimethyl labeling, which introduce isotopes at the peptide level by using a chemical reaction with isotopic reagents. Dimethyl labeling is widely applied in the proteome quantification of various types of samples due to its high labeling efficiency, low cost, and high quantification accuracy. Compared to genomic analyses, the proteomics behind the molecular pathogenesis of human HCC is still inadequately studied. The complexity and high dynamic range of protein samples extracted from HCC tissues greatly compromise the performance of large-scale proteome quantification. A lot of new technologies and methods of LC separation and prefractation have been developed for proteome analysis in recent years. We have developed an online strong cation exchange−reversed-phase two-dimensional liquid chromatog-
raphy system (SCX−RP 2D LC) and a high/low pH RP−RP offline 2D LC strategy for high-performance proteome and phosphoproteome analyses in previous works.11−13 The SCX−RP 2D system exhibited good separation performance and high detection sensitivity due to the high orthogonality between SCX monolithic and RP separation materials. However, the sample loading capacity is limited due to the usage of the capillary SCX column for both sample loading and prefractionating. For the high/low pH RP−RP strategy, the orthogonality could be highly improved by the high/low pH 2D LC separation. However, only pH 7.5 was applied in the RP−RP strategy because of the silica-based RP materials that were utilized and because separation buffers with higher pH may destroy the RP materials.

In this study, a simple reversed-phase−strong cation exchange−reversed-phase three-dimensional liquid chromatography strategy (RP−SCX−RP 3D LC) was developed by combining on-column sample purification, stable isotope dimethyl labeling, and prefractionation at high pH by using a 5 mm × 35 mm (inner diameter × length) polystyrene-divinylbenzene (PS-DVB) beads-packed solid-phase extraction (SPE) column with online SCX−RP two-dimensional LC−MS/MS analysis. Separation buffer with high pH (>12, with 10% NH₃⋅H₂O) was utilized for offline prefractionation to increase the orthogonality between the high/low pH LC separations. This strategy was successfully applied to investigate the differential proteome expression of HCC and normal liver tissues, in which 2759 proteins were reliably quantified; in-depth analysis revealed that the HCC proteome is systematically dysregulated during the hepatocarcinogenesis process.

## EXPERIMENTAL SECTION

### Reagents and Materials

PS-DVB copolymer microparticles (60 μm, 300 Å) were obtained from Sepax ( Suzhou, China). Daisogel ODS-AQ (3 μm, 120 Å) was purchased from DAISO Chemical Co., Ltd. (Osaka, Japan). Formic acid (FA) and sodium cyanoborohydride (NaBH₃CN) were provided by Fluka (Buchs, Germany). All other chemicals and reagents were purchased from Sigma (St. Louis, MO, USA). Fused silica capillaries with 75 and 200 μm i.d. were obtained from Polymicro Technologies (Phoenix, AZ, USA). Acetonitrile (ACN, HPLC grade) was purchased from Merck (Darmstadt, Germany). All water used in experiments was purified using a Milli-Q System (Millipore, Bedford, MA, USA).

### Protein Extraction and Digestion

The HCC and normal human liver samples were provided by Eastern Hepatobiliary Surgery Hospital (Shanghai, China), and the study was approved by the Institutional Review Board of the hospital. Informed consent was obtained from patients enrolled in this study. The normal human liver tissues were the noncancerous tissues ≥2 cm outside the hepatic cancer nodules, whereas the HCC tissues were obtained from advanced stage HCC patients and were removed by surgery. The noncancerous liver tissue has been verified by histopathological examination, which excluded the presence of invading or microscopic metastatic cancer cells. The corresponding tissues were pooled together to minimize the interindividual differences.

The liver tissues were lysed in an ice-cold homogenization buffer containing 8 M urea, 50 mM Tris-HCl (pH 7.5), 1% Triton X-100 (v/v), 65 mM DTT, 1 mM EDTA, 0.5 mM EGTA, 1 mM PMSF, 100 μL of protease inhibitor cocktail per 10 mL of homogenization buffer, and phosphatase inhibitor (1 mM NaF, 1 mM Na₂VO₃, 1 mM C₆H₇Na₃O₆P, 10 mM NaO₂P₂). After being resuspended in 8 M urea and 100 mM triethylammonium bicarbonate (TEAB, pH 8.0), protein concentrations were measured by Bradford assay. Proteins were reduced with 5 mM DTT at 37 °C for 2 h and alkylated with 10 mM iodoacetamide in the dark at room temperature for 40 min. The sample solutions were diluted five times with 100 mM TEAB (pH 8.0) buffer to reduce the urea to <2 M and digested overnight with TPCK-treated trypsin at an enzyme-to-protein ratio of 1:25 (w/w). The resulting tryptic digests were stored at −80 °C before usage.

### Online SCX−RP LC Separation and MS Detection

An Accela 600 HPLC system (Thermo, San Jose, CA) consisting of a degasser and a quaternary surveyor MS pump was used. Peptides were separated on a 15 cm length fused silica capillary column (75 μm i.d.) packed in-house with C18 particles (3 μm, 120 Å). The RP separation was carried out by a 200 min gradient from 10 to 35% of ACN (v/v) in 30 μL of buffer B (ACN/0.1% FA) at a 200 nL/min flow rate followed by a wash reaching 80% buffer B. The automated sample injection and multidimensional separation using the SCX−RP system were performed as previously reported.12 Each tryptic digest was repeatedly successively collected, lyophilized, and stored at −80 °C before use.
peptides from the SCX segment to the C18 separation column. Each salt step lasted for 6 min except for the last two, which lasted for 10 min followed by a 15 min equilibration with buffer A. Finally, a 200 min binary RP gradient nanoflow LC−MS/MS analysis, as described above, was applied to separate the peptides in each cycle.

The LTQ-Orbitrap Velos mass spectrometer (Thermo, San Jose, CA) was operated in data-dependent mode. The spray voltage was 2.0 kV, with the ion transfer tube at 250 °C. The full mass scan acquired in the Orbitrap mass analyzer was from m/z 400 to 2000 with a resolution of 60 000. The 20 most intense peaks with charge state ≥2 and an intensity threshold of 300 were selected for fragmentation in the ion trap via collision-induced dissociation (CID). The dynamic exclusion was set as follows: repeat count, 1; repeat duration, 30 s; and exclusion duration, 90 s. System controlling and data collection were carried out by Xcalibur software, version 2.1 (Thermo).

Data Analysis

MS data acquired were processed using MaxQuant (version 1.1.1.36, http://www.maxquant.org/) using Andromeda as the search engine against the International Protein Index (IPI) Human, version 3.68, database (87 083 entries) with a precursor mass tolerance of 7 ppm and fragment mass deviation of 0.5 Da. The search included variable modifications of methionine oxidation and N-terminal acetylation and fixed modification of cysteine carbamidomethylation. Trypsin was set as the specific proteolytic enzyme. Peptides with minimum of six amino acids and a maximum of two missed cleavages were allowed for the analysis. For peptide and protein identification, the cutoff false discovery rates (FDR) were both set at 0.01. Triplets were selected as the quantification mode with the dimethyl Lys 0 and N-term 0 as the light labels, dimethyl Lys 4 and N-term 4 as the intermediate labels, and dimethyl Lys 8 and N-term 8 as the heavy labels. Default settings were used for all other parameters in MaxQuant. The protein interaction network analyses were performed by Ingenuity Systems Pathway Analysis software (IPA version 8.8, Ingenuity Systems Inc.).

RESULTS AND DISCUSSION

Performance of Offline High/Low pH RP−RP 2D System for Proteome Analysis

SCX coupled with RP chromatography is widely utilized for online or offline 2D LC separation due to the good orthogonality between these two separation modes. However, RP−RP 2D LC separation with high/low pH exhibits comparable or even better orthogonality and separation capability than that of the SCX−RP system, especially for analytes with low retention capability on the SCX adsorbents, such as phosphopeptides. The orthogonality of RP−RP 2D LC separation depends on the pH value of the mobile phase. Usually, the mobile-phase pH of the first and second dimensions is ~10 and 3, respectively. Further increasing the mobile-phase pH in the first RP dimension will destroy the silica-based RP adsorbents, which limits the orthogonality improvement of the 2D LC system. Therefore, the PS-DVB polymer-based adsorbent with a much higher stability in a wide pH range was applied for sample prefractionation in the first dimension, and the buffer pH was >12 (10% NH₃·H₂O) in our experiment.

A RP SPE column packed with PS-DVB beads was prepared to facilitate the purification and prefractionation of protein samples at high pH, as described in the Experimental Section. First, 100 μg of the tryptic digest of the protein sample extracted from normal human liver tissues was loaded onto the PS-DVB SPE column followed by sample purification with a 0.1% formic acid (FA) aqueous solution. Then, a series of elutions with 10, 15, 20, 25, 30, 35, 40, 50, 60, and 80% ACN aqueous solutions (v/v) containing 10% NH₃·H₂O was applied to fractionate the purified peptides to 10 fractions. After
adjusting the pH to \( \sim 3 \), the 10 fractions were lyophilized and redissolved in 0.1% FA solution, and each fraction was analyzed by nanoflow LC–MS/MS with a 90 min separation gradient. The SCX–RP online 2D LC separation with a series of stepwise elutions of 50, 100, 150, 200, 250, 300, 350, 400, 500, and 1000 mM NH\(_4\)AC (pH 2.7) was also performed for comparison. The total analysis time of both 2D analyses was 20 h. Finally, 4068 and 4078 unique peptides and 1623 and 1544 distinct proteins were identified by the RP–RP and SCX–RP 2D LC–MS/MS systems, respectively (Supporting Information SI–S1 and Tables S1 and S2). Therefore, this high/low pH RP–RP 2D strategy exhibited comparable performance in comprehensive proteome analysis to that of the SCX–RP 2D system.

**Comprehensive Proteome Quantification by Combining On-column Dimethyl Labeling and RP–SCX–RP 3D LC–MS/MS Analysis**

Dimethyl labeling is one of the most commonly used stable isotope labeling methods due to its high labeling efficiency and high quantification accuracy. Triplex isotope labeling could be achieved by combining CH\(_2\)O, CD\(_2\)O, and 13CD\(_2\)O with NaBH\(_3\)CN and NaBD\(_3\)CN, and all of the primary amine groups in the N-terminus of the peptides and side chain of lysines are isotopically dimethylated with a mass difference of 4 Da.\(^{15}\) In this study, a homemade RP–SPE column packed with 200 mg of PS-DVB material was utilized for the purification of tryptic digests, stable isotope dimethyl labeling, and high pH RP prefracionation of the proteins samples extracted from HCC or normal liver tissues. Each fraction collected from the high pH RP prefracionation was further analyzed by the SCX–RP online 2D LC–MS/MS system.

In our previous work, a pseudo-triplex dimethyl isotope labeling approach was established by labeling two identical samples with light and heavy isotopes, respectively, while the comparative sample is labeled with the intermediate isotope. Thus, this approach allows two replicated quantification results to be obtained in just one experiment, and the quantification accuracy can be controlled by setting a coefficient of variation (CV) criterion.\(^{12,16}\) This pseudo-triplex dimethyl isotope labeling approach was also utilized in this work. Identical tryptic digests of a protein sample extracted from normal human liver (100 \( \mu \)g) were labeled with light and heavy isotopes, respectively, while tryptic digest of a protein sample extracted from HCC human liver (100 \( \mu \)g) was labeled with intermediate isotope. After sequential triplex dimethyl labeling onto the PS-DVB SPE column, three fractions were collected from the RP–SPE column by using 18, 26, and 80% ACN aqueous solutions containing 10% NH\(_3\)H\(_2\)O. Each fraction was then analyzed by an online SCX–RP 2D LC–MS/MS system as described in the Experimental Section (Figure 1). All of the data sets were processed by MaxQuant with a FDR < 1% for both peptide and protein identification. Finally, all of the quantified peptides were filtered by CV < 50% in replicate quantification, and 2759 proteins were reliably quantified (Supporting Information SI–S2).

It can be seen that most of the quantified proteins came from the cytoplasm and belong to enzyme classification (Supporting Information SI–S1 and Figure S1). Many important enzymes were significantly changed in HCC tissues, such as the 33-fold downregulation of N-acetyltransferase 2 (NAT2), which is involved in the metabolic activation and detoxification of aromatic amines (potential hepatocarcinogens) (Figure 2). Aldo-keto reductase family 1, member B10 (AKR1B10), involved in reducing aliphatic and aromatic aldehydes, was upregulated 80-fold (Figure 2), which was consistent with a previous report that the expression of AKR1B10 is highly specific to HCC and that its inhibition suppressed tumor growth.\(^{17}\) Other significantly downregulated enzymes include aldehyde dehydrogenase 1 family, member A2 (ALDH1A2, −11-fold), hydroxysteroid (17-beta) dehydrogenase (HSD17B, −10-fold), aldo-keto reductase family 7, member A3 (AKR7A3, −9-fold), and cytochrome P450, family 3, subfamily A, polypeptide 4 (CYP3A4, −7-fold). Significantly upregulated enzymes include stearoyl-CoA desaturase (SCD, 26-fold), acyl-CoA synthetase long-chain family member 4 (ACSL4, 17-fold), branched chain amino-acid transaminase 2 (BCAT2, 16-fold), flap structure-specific endonuclease 1 (FEN1, 14-fold), and fatty acid desaturase 2 (FADS2, 12-fold) (Figure 2).
Among all of the quantified proteins, 648 proteins (Supporting Information SI−S2) showed changes in their relative ratios of more than 3-fold (HCC/normal). The top 5 related biological functions of these proteins are cancer, lipid metabolism, small molecule biochemistry, amino acid metabolism, and developmental disorder, which all have a close relationship with HCC pathology (Supporting Information SI−S2). The proteins that are related to protein synthesis, gene expression, and post-translational modification are shown in Figure 3 as a protein interaction network, and all of these proteins are significantly upregulated. Sequestosome 1 (SQSTM1) binds ubiquitin and regulates the activation of the nuclear factor kappa-B (NF-κB) signaling pathway,18 and it is upregulated 12-fold in the HCC sample. NF-κB is a family of transcription factors that serve as important regulators of the host immune and inflammatory responses.19 Therefore, significant upregulation of SQSTM1 might increase the inflammatory response during the progression of HCC pathology. On the other hand, SOSTM1 is also involved in ubiquitin proteasome degradation for substrates being transported for proteasomal degradation,20 and its significant upregulation reveals that the protein degradation pathway is activated in HCC tissue. Eukaryotic initiation factors (eIF) are proteins involved in the initiation phase of eukaryotic translation, and eIF3 is the largest scaffolding initiation factor in mammals and plays a central role in the initiation pathway. The seven subunits of eIF3 were reliably quantified in our experiments, and all of them were significantly upregulated, which implies that protein synthesis is also activated during the progression of HCC. This is also consistent with previous reports that implicated eIF3 in oncogenesis and in the maintenance of the cancerous state and that revealed the overexpression of eIF3 mRNA in various types of carcinomas.21,22

It has been widely reported that lipid metabolism is aberrant in the development of HCC, which was revealed by investigations in genomics, proteomics, and metabolomics.23,24 The interaction network of proteins related to lipid metabolism

Figure 3. Protein interaction network of proteins involved in protein synthesis, gene expression, and post-translational modification. Red indicates the upregulated proteins. The color depth indicates the magnitude of the change at the protein expression level. The shapes indicate the molecular classes. Lines connecting the molecules indicate molecular relationships. The solid lines indicate direct known interactions.
is shown in Figure 4. It can be seen that two fatty acid desaturases, FADS1 and FADS2, enzymes involved in unsaturated fatty acid biosynthesis, are both significantly upregulated in the HCC sample (7-fold and 12-fold, respectively). Hepatocyte nuclear factor 4α (HNF4α) is a member of nuclear receptor superfamily of ligand-dependent transcription factors that is enriched in liver tissue. It was reported that transient inhibition of HNF4α initiated hepatocellular transformation and that HNF4α was downregulated in liver cancer relative to normal tissues by total RNA analyses. Although HNF4α has not been quantified in our experiment, it can be observed that most of the proteins related to HNF4α are upregulated in this interaction network, such as cytochrome P450, family 3, subfamily A, polypeptide 7 (CYP3A7), enoyl-CoA delta isomerase 2 (ECI2), APOBEC1 complementation factor (A1CF), and others, except for cytochrome P450, family 3, subfamily A, polypeptide 4 (CYP3A4) and glycine-N-acyltransferase (GLYAT). The cytochrome P450 proteins are monoxygenases that catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids, and other lipids. Interestingly, the members of the cytochrome P450 family are differently regulated in our quantification results. Briefly, CYP4X1, CYP1A2, and CYP3A4 are downregulated; in contrast,
CYP3A7 and CYP51A1 are upregulated. Similar results were also reported by large-scale gene expression profiles and immunohistochemistry.25,26

A data-independent acquisition SWATH MS label-free protein quantification method was applied for HCC (10 cases) and normal (10 cases) individual liver sample analyses to evaluate the accuracy of large-scale proteome quantification by using dimethyl isotope labeling and to check the biological variation across various samples. The quantification results of 10 upregulated and 10 downregulated proteins are shown in Figure 5. It can be seen that the average ratios in the SWATH MS analysis were all consistent with the results obtained by dimethyl isotope labeling for pooled samples (Supporting Information SI−S5). However, there are indeed some variations between different samples, such as AKR1B10 being significantly upregulated in 7 of 10 HCC samples (3 HCC samples showed no changes).

**Phosphoproteome Dysregulation of Human HCC Liver**

The comparative phosphoproteome quantification of the normal and HCC samples was also performed in our previous work by a pseudo-triplex dimethyl isotope labeling approach.12 The data sets were reanalyzed by using MaxQuant, and 2307 phosphorylation sites from 1264 phosphoproteins were reliably quantified after controlling the CV < 50% in replicate quantification (Supporting Information SI−S3). The non-phosphorylated counterparts of 445 phosphoproteins were also quantified in this work by the RP−SCX−RP 3D LC−MS/MS system, corresponding to 983 phosphorylation sites (Supporting Information SI−S4). It can be seen that most of the phosphorylation sites have a similar quantitative ratio (HCC/normal) to that of the corresponding nonphosphoproteins. This means that the phosphorylation occupancy for most phosphoproteins is not changed and that the changes at phosphorylation sites are derived from the changes in the total protein expression level.27 Long-chain acyl-CoA synthetase 4 (ACSL4) was upregulated 17-fold, and its phosphorylation site, S674, was upregulated 23-fold. This does not exhibit a significant difference between its phosphorylated and total protein expression levels. Therefore, it is important to investigate the phosphorylation sites that have different expression trends from that of their corresponding non-phosphoproteins, which may act in important roles in the progression of HCC. Compared with the protein quantification results, 337 (34%) of the phosphorylation sites exhibit significantly different expression trends in replicate analyses, with p < 0.05 in Student’s t test (Supporting Information SI−S4 and Table S3). For example, myopodin (SYNPO2) is a tumor suppressor gene that plays roles in suppressing cell growth and motility, but the mechanism for its role in tumor suppression is
not clear. It was reported that integrin-linked kinase (ILK) interacts with SYNPO2 as well as increases both serine and threonine phosphorylation of myopodin protein, which activates SYNPO2 to suppress cell growth and motility in prostate cancer cells.28 Both SYNPO2 and ILK were significantly downregulated (−4.8-fold and −4.3-fold, respectively) in HCC tissue. Eight phosphorylated serine (pS) sites were reliably quantified, as shown in Table 1, and four pS sites were significantly downregulated in HCC tissue (from −4.8-fold to −2.4-fold), similar to what was observed for their protein expression. Interestingly, the pS264 site was significantly upregulated (3.5-fold) in HCC tissue. Therefore, our results provide a clear polymorphism, phosphorylation for SYNPO2 in cancer tissue, which might be important for elucidating the activation mechanism of SYNPO2 in tumor suppression. Signal transducer and activator of transcription 3 (STAT3) performs signal transduction and activation of transcription functions. It is activated by tyrosine phosphorylation to form homo- or heterodimers that translocate to the nucleus, where it acts as a transcriptional activator. Although STAT3 is thought to promote oncogenesis in various tissues, the tumor-suppressive function of STAT3 has also been reported in a brain tumor.29 30 In our quantification results, STAT3 was upregulated in HCC tissue (1.8-fold) at the protein level, but the pY705 site was significantly downregulated (−3.8-fold). This means that STAT3 is inhibited in HCC tissue and that STAT3 might also act as a suppressor in the progression of HCC. Extracellular signal-regulated kinases 1 and 2 (ERK1/2) are involved in a wide variety of cellular processes such as proliferation, differentiation, transcription regulation, and development. After activation by the phosphorylation of upstream kinases, ERK1/2 can translocate to the nucleus of stimulated cells, where they phosphorylate nuclear targets. We observed that the ERK2 protein was significantly upregulated in HCC tissue (4.9-fold) but the phosphorylation at pT185 sites was downregulated (−1.8-fold). The pY204 phosphorylation sites of ERK1 also exhibited a more obvious downregulation trend compared to that of ERK1 protein (−9.2-fold for pY204 and −2.4-fold for ERK1). This might be because phosphorylated ERK1/2 exhibit antiproliferative effects and their ratio will decrease during the progression of HCC, which is similar to the longitudinal progression of human prostate cancer.30

Although many technologies based on genomic, proteomic, and metabolomic analyses have been developed in recent years, elucidating the molecular mechanisms within the progression of HCC is still a challenge. This greatly hinders the prediagnosis and treatment of worldwide liver cancer. Dong et al. systematically investigated the genome expression profile of HCC tissue by using the serial analysis of gene expression (SAGE) approach, and 224 genes were identified as being differentially expressed in HCC tissue compared to their levels in normal liver tissue.26 The top biological functions related to these genes included genetic disorder, cancer, and lipid metabolism, which are similar to our results obtained in prostate quantification. Acute phase response signaling is activated during tissue injury, infection, or inflammation, and the gene expression of albumin, AMBP, APOA1/2, TTR, LBP, FGA, and FGB all show significant differential expression. However, at the protein expression level, only FGA and FGB showed similar significant trends, and other proteins demonstrate no significant changes (Supporting Information S2).

Although the proteome is product of both the transcription and translation of the genome, there is indeed some difference between these two levels. Therefore, the protein expression profile is essential to elucidate the molecular mechanism of HCC pathologic. Tan et al. studied the metabolic profile of HCC serum and discovered taurocholic acid, lysophosphatidylcholine (LPC) 22.5 as potential HCC diagnosis markers.31 They also observed that most of detected LPE and LPC species exhibited upregulation trends. LPE and LPC are derived from PE and PC by phospholipase A2 (PLA2) catalysis, and PLA2 is upregulated 3-fold in protein expression in our results, which may be one of the reasons for LPE and LPC upregulation. It was also reported that the PLA2 is activated after phosphorylation at its S505 site, after which it translocates to the membrane to begin catalysis.32 Interestingly, another four phosphorylation sites of cPLA2 (cystolic phospholipase A2) that were quantified in our phosphoproteome analysis all showed significant downregulation [pS434 (−3.0-fold), pS435 (−3.4-fold), pS437 (−3.4-fold), and pS729 (−5.5-fold)]. Therefore, these four phosphorylation sites might have less influence on cPLA2 activation.

### CONCLUSIONS

In this study, a RP–SCX–RP 3D LC–MS/MS strategy was developed and combined with a pseudo-triplex stable isotope dimethyl labeling approach for the proteome quantification of HCC and normal liver tissues. Although the resolution of the SPE column-based prefractionation cannot be as high as that of
the LC system, the PS-DVB SPE column provides a robust, time-saving, simple-to-operate, and high-throughput strategy for sample preparation in quantitative proteomics. Because all of the sample preparation procedures, such as sample purification, isotope dimethyl labeling, and high pH prefractionation, were all performed on the same PS-DVB SPE column, sample loss and unexpected contamination were greatly eliminated, which is extremely important for highly accurate proteome quantification. Finally, 2759 proteins were reliably quantified with CV < 50% in replicate quantification. Our work provides the largest quantitative proteomic data set of human HCC tissue to date, as well as gives an interesting overlap with the phosphoprotein quantification results obtained in our previous work, which may help to elucidate the molecular mechanisms of HCC progression.

**ASSOCIATED CONTENT**

* Supporting Information

Table S1: performance of the off-line high/low pH RP–RP 2D system in proteome analysis. Table S2: performance of the online SCX–RP 2D system in proteome analysis. Figure S1: cellular location and molecular type of the annotated quantified proteins. Figure S2: overlap of the identified unique peptides by 18, 26, and 80% ACN fractions, respectively. SI–S2: list of the 2759 reliably quantified proteins including 648 proteins significantly dysregulated by more than 3-fold in HCC tissue. SI–S3: list of the 2307 phosphorylation sites corresponding to 1264 phosphoproteins obtained in previous quantitative phosphoproteome analysis. SI–S4: list of the 445 phosphoproteins with 983 phosphorylation sites whose nonphosphorylated counterparts were reliably quantified in the proteome analysis. SI–S5: quantification results of 10 upregulated and 10 downregulated proteins in data-independent acquisition SWATH MS label-free protein quantification for HCC (10 cases) and normal (10 cases) individual liver sample. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

The authors declare no competing financial interest.

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**REFERENCES**


(16) Wang, F.; Blanchard, A. P.; Elisma, F.; Granger, M.; Xu, H.; Bennett, S. A. L.; Figeys, D.; Zou, H. Phosphoproteome analysis of an early onset mouse model (TgCRND8) of Alzheimer’s disease reveals...
temporal changes in neuronal and glia signaling pathways. Proteomics 2013, 13, 1292–1305.


