Comparative analysis of primary hepatocellular carcinoma with single and multiple lesions by iTRAQ-based quantitative proteomics

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors, which is causing the second leading cancer-related death worldwide [1,2]. Approximately 748,000 new cases occur worldwide per year according to the 2014 investigation of International Agency for Research on Cancer (IARC) [3]. Although surgical excision was demonstrated to be the first choice for HCC treatment according to the clinic experiences of the past 50 years, the prognosis of HCC patients is still unsatisfactory [4–7]. Many factors, which could affect the prognosis of the surgical treatment of HCC patients, have been reported in previous studies; among them, tumor size, tumor lesion number and portal vein tumor thrombus (PVTT) were reported to be the most important factors [8–12].

Tumor lesion number, as one of the most intuitive and evident tumor pathological characteristics, has a very important impact on the treatment and prognosis of HCC patients [13–17]. Ikeda et al. have studied the prognosis of 83 HCC patients treated with curative surgical resection, and they showed that the total number of tumor nodules was one of the most significant factors affecting the tumor recurrence and overall survival time of patients [18]. Imanura et al. have epidemiologically shown that multiple tumor lesion number was one of the most important contributors to the late phase recurrence of HCC patients [19]. Furthermore, the tumor lesion number is one of the key factors affecting the tumor TNM stage, and multiple tumor lesion numbers always reflect higher TNM stage, which has a significantly worse prognosis [20]. Therefore, the tumor lesion number did play a very important role in the prognosis of HCC. However, the underlying molecular mechanisms of the tumorigenesis, progress and prognosis of the primary HCC with single and multiple observed lesions are still unclear.

The high-throughput quantitative proteomic technologies offered the way to systematically characterize the overall proteome alterations under any physiological or pathological changes, which could provide fundamental information for the complicated diseases. Particularly, the isobaric tags for relative and absolute quantitation (iTRAQ) profiling technology enables the quantitative and direct comparison of the proteome changes up to 8 samples simultaneously with very high sensitivity and protein coverage [21,22]. Several groups have reported the
application of iTRAQ-based quantitative proteomics approach in the study of hepatic cancer, especially for the screening of diagnostic and prognostic protein biomarkers [23–25]. For instance, Huang et al. have reported the iTRAQ-based quantitative analysis of the recurrence/metastasis of HCC (diameter is larger than 10 cm) at different stages, and identified two biomarkers for distinguishing and predicting the early recurrence/metastasis behaviors of HCC [26]; Wang et al. have reported the serum proteome of metastatic HCC using the iTRAQ-based quantitative analysis and identified a metastasis-associated biomarker [27]. Ko et al. have reported the iTRAQ-based quantitative analysis of HCC cancer stem cell proteome [28]. Meanwhile, the iTRAQ-based quantitative study of the proteome changes and the secreted proteome changes during HBV infection have also been reported [29,30]. However, the application of iTRAQ labeling in studying the molecular differences between the primary HCC with a single lesion and the primary HCC with multiple lesions has never been reported.

Here we quantitatively compared the proteomes of the tumor tissues and the adjacent tissues obtained from the primary HCC patients with single and multiple observed lesions using iTRAQ-based quantitative proteomic approach (2D LC–MS/MS), and identified potential biomarkers for distinguishing the primary HCC with single and multiple lesions.

2. Materials and methods

2.1. Sample collection

Tissue samples, including the cancerous and surrounding non-cancerous tissues, were obtained from 30 primary HCC patients with multiple observed lesions and 30 primary HCC patients with a single observed lesion, respectively. All patients have undergone radical surgery at Mengchao Hepatobiliary Hospital of Fujian Medical University from August 2010 to January 2013. The absence of intrahepatic recurrence/metastasis in the residual liver was monitored by ultrasonography (US), computer tomography (CT) scan and angiography. All of the patients followed the Enrollment Eligibility Criteria: (1) the patient was diagnosed with HCC by post-operative pathological examinations; (2) pre-operative serum HBsAg (Hepatitis B surface antigen) positive, but HbcAb (Hepatitis B core antibody) negative; (3) subject to the standard radical resection [7]; no distal metastasis was revealed in both pre- and intra-operative examinations; no lesion was found in the rest of the liver during intra-operative ultrasonic scan; no visible cancer embolus in the hepatic portal vein or primary venous branch; no cancer cell was found in the incisal margin at the post-operative pathological examinations; no recurrent/metastatic lesion was found at the ultrasonic and CT scan during the return visit after 2 months of surgery; (4) the elevated pre-operative serum AFP should decline to normal level after 2 months of post operation; and (5) the patient did not undergo any other intervention or therapies before surgery.

Fresh tissues were collected at the time of surgery from patients with Hepatitis B virus (HBV) associated primary HCC; part of the collected tissues was immediately liquid nitrogen preserved after washing with phosphate-buffered saline (PBS), and part of the tissues was formalin embedded and stored for immunohistochemistry. The histological diagnosis of the tissue samples was confirmed by experienced pathologists. The project was approved for the use of human biopsy by the Institution Review Board of Mengchao Hepatobiliary Hospital of Fujian Medical University. The written consent was received from all participants in this study.

2.2. Protein preparation and iTRAQ labeling

The tissues from patients were divided into 4 groups: cancerous tissues from HCC patients with multiple observed lesions (MC group, n = 30); surrounding non-cancerous tissues from HCC patients with multiple observed lesions (MN group, n = 30); cancerous tissues from primary HCC patients with a single observed lesion (SC group, n = 30); surrounding non-cancerous tissues from primary HCC patients with a single observed lesion (SN group, n = 30). For each group, every 5 individual samples with equal tissue weight were mixed (for the patient groups with multiple observed lesions, we only collected the largest tumor specimen from multiple lesions of each individual, then mixed equal tumor tissue weight of 5 individual samples), and then the proteins were extracted from the mixed samples. We have 6 repeat- ed protein extracts for each group to minimize the individual differences of the patients.

One milliliter of lysis buffer containing 8 M urea, 2% SDS and 1× protease inhibitor cocktail (Roche Ltd. Basel, Switzerland) were added to the mixed samples, then followed by tissue homogenization and sonication on ice. After centrifugation at 17,000 g for 10 min at 4 °C, the supernatant was collected and transferred to a fresh tube. The protein concentration of the supernatant was determined by BCA assay (TransGen Biotech, Beijing, China) following the manufacturer’s protocol. Afterwards, 100 μg proteins per condition were transferred into a new tube, and the final volume was adjusted to 100 μl with 100 mM TEAB (triethylammonium bicarbonate). Then 5 μl DTT (200 mM) was added into the protein samples, and the samples were further incubated at 55 °C for another 1 h; afterwards, 10 μl iodoacetamide (500 mM) was added to each sample to alkylate the proteins, then all of the samples were incubated for 30 min in dark at room temperature.

For each sample, proteins were precipitated by ice-cold acetone, and then were re-dissolved in 100 μl TEAB (100 mM). Afterwards, the proteins were typically digested by sequence-grade modified trypsin (Promega, Madison, WI), and then the resultant peptide mixture was further labeled using chemicals from the iTRAQ reagent kit (AB SCIEX, USA). Peptides were labeled with the iTRAQ 8-plex reagent as follows: four groups (MC group, MN group, SC group and SN group) were labeled with 113, 114, 115 and 116 isobaric tags, respectively; and the peptides from the biological repetitions of the above 4 groups were labeled with 117, 118, 119 and 121, respectively. The iTRAQ 8-plex labeling was independently repeated 3 times, defining as A, B and C. Equal amounts of labeled samples were desalted with the Sep-Pak Vac C18 cartridges and then dried in a vacuum centrifuge for further usage.

2.3. High pH reverse phase separation

The peptide mixture was re-dissolved in the solution A (solution A: 20 mM ammonium formate in water, the pH was adjusted to 10.0 with ammonium hydroxide), and then fractionated by high pH separation using an Acquity UPLC system (Waters Corporation, Milford, MA) connected to a reverse phase column (BEH C18, 1.7 μm, 21 × 50 mm, Waters Corporation, Milford, MA). High pH separation was performed using a linear gradient starting from 5% B to 35% B in 20 min (solution B: 20 mM ammonium formate in 90% ACN, the pH was adjusted to 10.0 with ammonium hydroxide). The column flow rate was maintained at 600 μl/min and column temperature was maintained at room temperature after the separation. The column was re-equilibrated at initial conditions for 15 min. Finally 40 fractions were collected, and two fractions with the same time interval were pooled together to reduce the fraction numbers, such as 1 and 21, 2 and 22, and so on [31]. Twenty fractions at the end were dried in a vacuum concentrator for further usage.

2.4. Low pH nano-LC–MS/MS analysis

The fractions were re-suspended with a 32 μl solution C (solution C: 0.1% formic acid in water), and separated by nano-LC and analyzed by on-line electrospray tandem mass spectrometry. The experiments were performed on a Nano-Acquity UPLC system (Waters Corporation, Milford, MA) connected to a quadrupole–orbitrap mass spectrometer (Q-Exactive) (Thermo Fisher Scientific, Bremen, Germany) equipped with an online nano-electrospray ion source. 8 μl peptide sample was loaded onto the trap column (Thermo Scientific Acclaim PepMap C18, 100 μm × 2 cm) with a flow of 10 μl/min, and subsequently separated on the analytical column (Acclaim PepMap C18, 75 μm × 50 cm) with
a linear gradient, from 2% D to 40% D in 135 min (solution D: 0.1% formic acid in ACN). The column flow rate was maintained at 300 nL/min and the column temperature was maintained at 40 °C. The electrospray voltage of 2.2 kV at the inlet of the mass spectrometer was used. After the nano-LC separation, the column was re-equilibrated at initial conditions for 15 min.

The Q-Exactive mass spectrometer was operated in the data-dependent mode to switch automatically between MS and MS/MS acquisitions. Survey full-scan MS spectra (m/z 350–1200) were acquired with a mass resolution of 70 K, followed by 15 sequential high energy collisional dissociation (HCD) MS/MS scans with a resolution of 17.5 K. In all cases, one microscan was recorded using dynamic exclusion of 30 s.

2.5. Data analysis

All the raw files generated by the Q-Exactive instrument were converted into mzXML and MGF files using the MS convert module in Trans-proteomic Pipeline (TPP 4.6.2). All MGF files were searched using Mascot (Matrix Science, London, UK; version 2.3.0) against a human database provided by The Universal Protein Resource (http://www.uniprot.org/uniprot, released at 2014-04-10, with 20,264 entries). The enzyme specificity of trypsin was used and maximally up to two missed cleavages were allowed for protease digestion. Mascots were searched with a parent ion tolerance of 10 parts per million (ppm) and a fragment ion mass tolerance of 0.05 Da. Carbamidomethylation of cysteine, as well as iTRAQ modification of peptide N-terminus and lysine residues were set as fixed modifications; oxidation of methionine and iTRAQ cation were set as variable modifications.

The differentially expressed proteins verified by qPCR were further confirmed by immunoblotting. The tissue samples were lysed in a protein extraction buffer (150 mM NaCl, 10 mM Tris, 5 mM EDTA, 1% Triton X-100, 5% glycerol, and 0.1% SDS, pH 7.2) containing protease inhibitor cocktail (Roche, Indianapolis) on ice. After centrifugation at 17,000 g for 30 min at 4 °C, the supernatant was collected and transferred to a fresh tube. The protein concentration of the supernatant was determined by BCA assay (TransGen Biotech, Beijing, China) following the manufacturer’s protocol. Then, 30 μg proteins of each sample were separated by SDS-PAGE and transferred onto the NC membranes (Millipore, Bedford, MA). Afterwards, the membranes were blocked for 2 h in the PBST buffer containing 5% BSA, and probed with the HSD17B13 and HK2 primary antibodies (1:5000 dilution, Abcam) and β-actin antibody (1:5000 dilution, TransGen Biotech) at 4 °C overnight. After they were washed 3 times with a PBST buffer for 10 min of each, the membranes were incubated with appropriate HRP-conjugated secondary antibodies (1:5000 dilution, TransGen Biotech) for 1 h at room temperature. Following washing again in the TBST buffer, the protein expression levels were detected by enhanced chemiluminescence and were visualized by autoradiography.

3. Results

3.1. Proteomic analysis of the primary HCC with single and multiple lesions

The quantitative MS-based discovery strategy was applied to study the overall proteome of human primary HCC with single and multiple lesions. The detailed characteristics of the selected HCC patients were listed in Table 1 of [32]. All of the patients were selected with similar age distributions, AFP levels, tumor size distributions, cirrhosis levels, differentiation degrees, degree of tumor boundary definition, degree of tumor encapsulation integrity, and similar vascular tumor thrombosis. Total proteins extracted from the collected tumors and their adjacent tissues from patients were analyzed using iTRAQ 2D LC–MS/MS, and the workflow was described in Fig. 1. The mix of samples as mentioned in the “Materials and methods” section could allow the reduction of the individual differences among patients.

3.2. Relative quantification of proteomes of the primary HCC with single and multiple lesions

Using the results from Scaffold_4.3.2, we quantified 5079, 5109, and 5222 proteins in three iTRAQ 8-plex labeling replicates respectively. And 4601 proteins were shared by the three replicates, accounting for 83.51% of the total quantified proteins. The number of overlapped proteins among 3 iTRAQ 8-plex labeling replicates was displayed by the Venn diagram in Fig. 2A. The complete list of identified proteins in our study was shown in Table S1 of [32]. The detailed characteristics of
proteomes of the primary HCC with single and multiple lesions, including molecular weight (MW), isoelectric point (PI), hydrophobicity, exponentially modified protein abundance index (emPAI), quantitative clustering, average coefficient of variance (CV), and quantification results with percentage variability, were included in the list as well. Full details of the raw dataset can be accessed through http://pan.baidu.com/s/1kT5ixO7.

The distribution of unique peptide numbers per protein showed that nearly 90% of the total quantified proteins had at least 2 unique peptides. The distribution of MW, PI and hydrophobicity also clearly showed that the overall proteome datasets of the primary HCC with single and multiple lesions had no strong bias (Fig. 1 in [32]). We also compared the abundance level of all the quantified proteins by calculating their exponentially modified protein abundance index (emPAI) [33]. Log10 of these emPAI are plotted in Fig. 2B, and the plots clearly show that their abundance have a wide span, which covers 7 orders of magnitude. Gene Ontology annotation was used to analyze the subcellular localizations of identified proteins. Although the cytoplasmic and membrane-associated proteins were the most highly represented categories in our extracts, the nuclear and extracellular proteins were also readily identified; it indicates that our protein extraction procedure was not strongly biased to a few cell compartments (Fig. 2C). The involved biological processes of the identified proteins were mostly enriched for the cellular metabolic process and organelle organization, which maintain many of the body's functions (Fig. 2D).

### 3.3. Differentially expressed proteins in the primary HCC with single and multiple lesions

In this study, 107 and 330 proteins were classified as differentially expressed in HCC tumor tissues with single and multiple lesions compared with surrounding non-cancerous tissues (Fig. 2A, B in [32]). All of the differentially expressed proteins presented a mean expression fold change of ±1.5 (log2 0.58) or even more with a p value of less than 0.05 (paired T-test); meanwhile these proteins should have the same change trends in all six biological replicates. The typical proteins that were differentially expressed in HCC with single and multiple lesions when compared with its adjacent non-cancerous tissue were listed in Table 1. Among these differentially expressed proteins, 71 proteins

<table>
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<tr>
<th>Proteins</th>
<th>Gene</th>
<th>Fold change MC/MN</th>
<th>Fold change SC/SN</th>
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<td>1.51</td>
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<tr>
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**Fig. 1.** Schematic view of the experimental design and the iTRAQ 8 plex-labeling. Sample preparation procedures for shotgun MS/MS analysis, and important steps in the proteomic strategies for the detection of dysregulated proteins in cancerous and non-cancerous HCC tissues. The tissues from the primary HCC patients with single and multiple lesions were divided into four groups: MC group, MN group, SC group and SN group. Proteins extracted from abovementioned four groups were further digested by trypsin and labeled by different iTRAQ reagents. Equal amounts of labeled peptide mixture were then analyzed by 2D LC–MS/MS. In the workflow, different-labeled samples were represented in different colors. For each sample, eluted proteins were digested, separated by high pH reversed phase LC, and analyzed by LC–MS/MS. For each experiment, iTRAQ tags were assigned to a duplicate in all 3 repeats: MC group (113, 117), MN group (114, 118), SC group (115, 119) and SN group (116, 121).
altered their expression in both HCC types (Fig. 2C in [32]). GO annotation analysis showed that these proteins were the major participants in the oxidation reduction process and the cellular metabolic processes, which is suggesting that the disorder of primary metabolism might be either a cause or effect of HCC development (Fig. 2D in [32]). Interestingly, there were 36 dysregulated proteins in MC group compared with MN group, but these proteins were not dysregulated in primary HCC with a single lesion (Fig. 2C in [32]). The quantitative iTRAQ ratios of these 36 proteins, which were averaged from six biological replicates, were plotted on a heatmap. It is shown that 13 proteins were significantly up-regulated and 23 proteins were significantly down-regulated in MC group compared with MN group, and these two types of proteins formed clearly distinct clusters (Fig. 3A in [32]). The names of the dysregulated proteins were listed in Table 2 of [32]. We further analyzed these protein involved biological process by GO analysis; the results showed that these dysregulated proteins were mainly involved in oxidation reduction, cataabolic and metabolic process of various acids (carboxylic acid, cellular amino acid, bile acid), amines, aromatic amino acid family, aromatic compounds and steroids (Fig. 3C in [32]). All of these processes belong to material metabolism, and illustrated that the primary material changes played an important role in the tumorigenesis and development of HCC with multiple lesions. Meanwhile, 142 up-regulated proteins and 117 down-regulated proteins specifically appeared in HCC with a single lesion group, but not in HCC with a multiple lesion group; and the up- and down-regulated proteins also form clearly distinct clusters in the heatmap (Fig. 3B in [32]). The list of protein names was also displayed in Table 3 in [32]. Interestingly, the enriched biological processes of dysregulated proteins in HCC with a single lesion mainly focused on oxidation reduction, oxidative phosphorylation, ATP synthesis and electron transport (Fig. 3D in [32]), which reflected a significant mitochondrial dysfunction. Although the most common change in biological process in both HCC with a single lesion and HCC with multiple lesions is oxidation reduction, there are still specific changes of the biological processes in these two HCC types, which is suggesting that there might be different molecular mechanisms in the carcinogenesis and development of the primary HCC with single and multiple lesions.

3.4. IPA network analysis of the differentially expressed proteins

To further reveal the possible molecular mechanisms of the tumorigenesis and development of the primary HCC with a different number of lesions, we applied the IPA software to analyze the signaling pathways that the differentially expressed proteins were involved. The results show that specific signaling pathways were indeed involved in the tumorigenesis and development of the primary HCC with single and multiple lesions, although there are common signaling pathways involved in as well. According to the IPA analysis, the dysregulated proteins in HCC with multiple lesions are mostly involved in UBC signaling pathway and NFκB signaling pathway, while the dysregulated proteins in HCC with a single lesion are mainly involved in ERK signaling pathway and NFκB signaling pathway.

In HCC with multiple lesions, 12 proteins (including 3 up-regulated proteins and 9 down-regulated proteins) were involved in the UBC signaling pathway; and 13 proteins (including 7 up-regulated proteins and
6 down-regulated proteins) were involved in NFκB signaling pathway (Fig. 3A). While in the HCC with a single lesion, 13 proteins (including 8 up-regulated proteins and 5 down-regulated proteins) were involved in the ERK signaling pathway; and 24 proteins (including 16 up-regulated proteins and 8 down-regulated proteins) were involved in NFκB signaling pathway (Fig. 3B). Although all of the abovementioned signaling pathways are actively associated with cancers [34–37], only the NFκB signaling pathway is commonly shared by both HCC types. It has been reported that the NFκB signaling pathway played an essential role in the development and aggressive behavior of tumor by enhancing tumor angiogenesis, proliferation, anti-apoptosis, and repressing immune responses [38,39]. Therefore, it is not surprising that the NFκB signaling pathway is involved in both groups. Interestingly, the UBC (polyubiquitin-C) signaling pathway is only enriched in the HCC with multiple lesions, but not enriched in the HCC with a single lesion; UBC signals play an essential role in the ubiquitin–proteasome proteolytic system in mammals, and precisely controls the protein degradation. These results suggested that the HCC with multiple observed lesions had more serious problems in protein degradation disorder. Although, the disturbance of protein degradation has been reported to be closely associated with different cancer types [40–43], the underlying mechanisms of the disturbance of UBC signaling pathway, which only occurred in HCC with multiple observed lesions, are not clear and need further investigation. On the other hand, the ERK signaling pathway is only enriched in the HCC with a single lesion, but it is normally regulated in HCC with multiple lesions; ERK signals were well known as an extremely important regulator of cell growth and proliferation. Therefore, the development of primary HCC with a single lesion might be closely associated with cell proliferation and growth; however, the changes of ERK signaling in different HCC types would be better analyzed and verified by phosphoproteomics, and the exact underlying mechanisms should be further studied.

The above analyzed results clearly proved that our quantitative proteomics approach is suitable to study the overall molecular profile changes of the different HCC types, and could give further deep insight into the possible molecular mechanisms.

3.5. The differentially expressed proteins in the primary HCC with single and multiple lesions

According to the pathway network analysis, we found that two proteins HSD17B13 and HK2 were differentially expressed between HCC with a single lesion and HCC with multiple lesions. HSD17B13 which is known as short-chain dehydrogenase/reductase 9 is a lipid droplet-associated protein [44]; and its down expression has been proven in human liver [45]. HK2 is a major isozyme that is involved in glycolysis, which played a key role in promoting anabolic pathways, and is frequently over-expressed in different cancers [56]. Therefore, they might be potential interesting biomarkers in distinguishing the HCC with a single lesion and HCC with multiple lesions. Here, we further

**Fig. 3.** The key signaling pathways involved in the HCC with single and multiple lesions. The top 2 involved signaling pathway in the HCC with multiple lesions (A, UBC signaling pathway and NFκB signaling pathway), and in the HCC with a single lesion (B, ERK signaling pathway and NFκB signaling pathway). The red labeling indicates the up-regulated proteins and green labeling indicates the down-regulated proteins.
verified the expression profile of these 2 proteins on the primary HCC patient samples with different numbers of tumor lesions, at MS/MS spectrum, mRNA and protein levels.

The relative intensities of the reporter ion of 8-plex iTRAQ reagent of these two proteins were checked in the MS/MS spectra. As shown in Fig. 4A, the expression levels of HSD17B13 were significantly lower in 113 and 117 labels (MC) than in 114 and 118 labels (MN) with an iTRAQ ratio of >1.5 and p value of <0.05. Instead, the expression levels of HK2 were significantly higher in 115 and 119 labels (SC) than in 116 and 121 labels (SN) with an iTRAQ ratio of <0.67 and a p value of <0.05.

Afterwards, we further performed the qPCR and Western blot to analyze the expression of these two proteins at the mRNA and protein levels. As shown in Fig. 4B, the mRNA expression of HSD17B13 was only significantly down-regulated in the group of primary HCC with multiple lesions (n = 93 patients, 2.8 fold down-regulated, p < 0.05), while the mRNA expression of HK2 was only significantly up-regulated in the primary HCC with a single lesion (n = 98 patients, 1.6 fold up-regulated, p < 0.05), compared with their surrounding non-cancerous tissues, respectively. This phenomena could be further confirmed at the protein level. As shown in Fig. 4B and C, the protein level of HSD17B13 was significantly down-regulated in the primary HCC with multiple lesions, while the protein level of HK2 was remarkably up-regulated in the primary HCC with a single lesion. These results were well consistent with the quantitative proteomics results. Therefore, the HSD17B13 and HK2 might be potential biomarkers for distinguishing the primary HCC with single or multiple lesions, but the underlying molecular mechanisms of these proteins should be further clarified.

4. Discussions

As reported in previous studies, tumor lesion was an important factor for the prognosis of HCC. In this study, quantitative comparison of the overall proteome between the primary HCC with a single lesion and HCC with multiple lesions was firstly performed. 5511 proteins with FDR < 1% were quantified in our experiments which is relatively high compared with other studies in liver cancer research [26,46]. Among those identified proteins, 83.51% of them were shared in all 3 biological repeats, which proved the stability of the workflow and the reliability of the research conclusion. There are significant differences between HCC with a single lesion and HCC with multiple lesions at molecular level through a comprehensive analysis of the differentially expressed proteins. The abnormal regulation of fatty, protein and various acid metabolism, which might lead to the significant change of the liver microenvironment, has been revealed in the primary HCC with multiple lesions. The tumor microenvironment (TME) is the soil to develop tumors, and has an extremely important role in the tumorigenesis, development and prognosis.

Fig. 4. Validation of the differentially expressed proteins in the primary HCC with single and multiple lesions. (A) The relative intensity of reporter ion of the 8-plex iTRAQ reagent regarding HSD17B13 and HK2 in MS/MS spectra. The typical spectrum represented that the expression levels of HSD17B13 were significantly lower in 113 and 117 labels (MC) than in 114 and 118 labels (MN). The typical spectrum represented that the expression levels of HK2 were remarkably higher in 115 and 119 labels (SC) than in 116 and 121 labels (SN). (B) The mRNA and protein levels of HSD17B13 and HK2. HSD17B13 are down-regulated in the tumor samples from the multiple HCC patients compared with their adjacent tissues (p < 0.05, paired T-test). HK2 are significantly up-regulated in the tumor samples from the single HCC patients compared with their adjacent tissues (p < 0.05, paired T-test). (C) The protein expression levels of HSD17B13 and HK2 when validated by Western blot.
of cancers [47,48]. The disorder of the liver environment could cause various problems, such as abnormal angiogenesis, destruction of extracellular matrix (ECM), and severe inflammation [49–53]. Therefore, the disorder of the TME might be one of the key factors for carcinogenesis and progression of the primary HCC with multiple lesions.

In contrast, the top enriched biological processes in the single HCC group focused on oxidative phosphorylation, ATP synthesis and electron transport, which mainly reflected the abnormal functions of the mitochondria. Oxidation phosphorylation and energy dysfunction, the main results of the mitochondrial dysfunction have been reported to be closely related to the development and progression of tumors and have been reported to be involved in many cancers [54–56]. Abnormal mitochondrial metabolism may lead to abnormal cellular homeostasis and energy imbalance [40]. The dysregulation of the energy production in the HCC with a single lesion might lead to increased proliferation ability than that in the HCC with multiple lesions, and therefore the single HCC tumors are most likely to develop into larger sizes. We carefully analyzed the clinical features of both HCC types from our tissue bank containing 79 HCC patients with multiple lesions and 78 HCC patients with a single lesion. From our tissue bank, the selected patients are all male and the average age of each group is quite similar (the age of HCC patients with multiple lesions is 53.51 ± 11.00 years, and the age of HCC patients with a single lesion is 52.08 ± 11.45 years), while the average tumor diameter of HCC with a single lesion (6.03 ± 1.77 cm) is significantly larger than HCC with multiple lesions (3.14 ± 2.21 cm). This phenomenon is well supporting our above conclusion, but we still need further studies to prove. Furthermore, this conclusion could be further supported by the obviously altered ERK signaling pathway in the HCC with a single lesion, which is linking to promote cell proliferation, increase tumor angiogenesis and induce tumor invasion.

The expression of HSD17B13 is significantly down-regulated in the HCC with multiple lesions, while its expression in the HCC with a single lesion has not been significantly changed. Recently, it has been reported that the expression of HSD17B13 was closely associated with the formation or development of tumors and the metabolism of adipose in liver [57–59]. In this study, we discovered and validated that HSD17B13 was down-regulated in the HCC patients with multiple lesions. The results suggest that HSD17B13 might play important roles in the initiation and progression of HCC with multiple lesions, and might be a potential biomarker for distinguishing the HCC with multiple lesions. Instead, the expression of hexokinase 2 (HK2) was significantly up-regulated in HCC with a single lesion, but was kept unchanged in the HCC with multiple lesions. Recent studies have demonstrated that the HK2 could promote glycolysis in tumor cells but not in normal cells [60,61], and increase the tumor progression and aggressive behaviors in many cancers [62–65]. Palmieri et al. have reported that the poor patient survival and the metastasis of breast cancer to the brain were significantly associated with high HK2 expression [66], and HK2 ablation even could inhibit the metastasis [61]. Therefore, the up-regulation of HK2 might associate with the larger tumor size and early tumor recurrence/metastasis features of the HCC with a single lesion as previously reported [19,67]. Of course, the metastasis and prognosis were affected by many factors, especially the stage of diagnosis and the strategies of therapy.

Overall, we have applied the iTRAQ-based quantitative proteomics approach to compare the protein expression profile alternations between HCC with a single lesion and HCC with multiple lesions, and identified potential biomarkers for distinguishing these 2 HCC types.

## 5. Conclusions

Here, we have applied the iTRAQ-based quantitative proteomics approach to study the overall protein profile alternations of the primary HCC with single and multiple lesions after radical resection. The results clearly proved that different protein profile alternations and different signaling pathways were involved in the primary HCC with single and multiple lesions. Meanwhile, we have identified and validated the proteins HSD17B13 and HK2 as potential interesting biomarkers for distinguishing these 2 HCC types.

## Abbreviations

- **HCC**: hepatocellular carcinoma
- **iTRAQ**: isobaric tags for relative and absolute quantification
- **IARC**: International Agency for Research on Cancer
- **PVTT**: portal vein tumor thrombus
- **TNM**: tumor node metastasis
- **2D LC–MS/MS**: two-dimensional liquid chromatography–tandem mass spectrometry
- **US**: ultrasonography
- **CT**: computer tomography
- **HbsAg**: hepatitis B surface antigen
- **HbcAb**: hepatitis B core antibody
- **AFP**: alpha fetoprotein
- **HBV**: hepatitis B virus
- **BCA**: bicinchoninic acid
- **TEAB**: triethylammonium bicarbonate
- **DTT**: DL-Dithiothreitol
- **IAA**: iodoacetamide
- **ACN**: acetonitrile
- **HCD**: high energy collisional dissociation
- **ppm**: parts per million
- **FDR**: false discovery rate
- **GO**: Gene Ontology
- **IPA**: ingenuity pathway analysis
- **MW**: molecular weight
- **PI**: isoelectric point
- **emPAI**: exponentially modified Protein Abundance Index
- **CV**: average coefficient of variance
- **TME**: tumor microenvironment
- **ECM**: extracellular matrix

## Transparency document

The Transparency documents associated with this article can be found, in online version.

## Acknowledgments

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## References


## Abbreviations

- **DTT**: DL-Dithiothreitol
- **TEAB**: triethylammonium bicarbonate
- **BCA**: bicinchoninic acid
- **HBV**: hepatitis B virus
- **HbcAb**: hepatitis B core antibody
- **HbsAg**: hepatitis B surface antigen
- **AFP**: alpha fetoprotein
- **HBV**: hepatitis B virus
- **BCA**: bicinchoninic acid
- **TEAB**: triethylammonium bicarbonate
- **DTT**: DL-Dithiothreitol
- **IAA**: iodoacetamide
- **ACN**: acetonitrile
- **HCD**: high energy collisional dissociation
- **ppm**: parts per million
- **FDR**: false discovery rate
- **GO**: Gene Ontology
- **IPA**: ingenuity pathway analysis
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