Journal of Proteomics 150 (2017) 230–241

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Reveal the molecular signatures of hepatocellular carcinoma with different sizes by iTRAQ based quantitative proteomics

Yingchao Wang [a,b], Hongzhi Liu [a,b,c], Dong Liang [a,b,c], Yao Huang [a,b,c], Yongyi Zeng [a,b,c], Xiaohua Xing [a,b], Jiangbao Xia [a,b], Minjie Lin [a,b], Xiao Han [d], Naishun Liao [a,b], Xiaolong Liu [a,b,*], Jingfeng Liu [a,b,c,*]

[a] The Liver Center of Fujian Province, Fujian Medical University, Fuzhou 350025, People's Republic of China
[b] The United Innovation of Mengchao Hepatobiliary Technology Key Laboratory of Fujian Province, Mengchao Hepatobiliary Hospital of Fujian Medical University, Fuzhou 350025, People's Republic of China
[c] Liver Disease Center, The First Affiliated Hospital of Fujian Medical University, Fuzhou 350007, People's Republic of China
[d] Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, People's Republic of China

ARTICLE INFO

Article history:
Received 30 March 2016
Received in revised form 3 August 2016
Accepted 19 September 2016
Available online 28 September 2016

Keywords:
Hepatocellular carcinoma
Quantitative proteomics
Tumor size
Tumor growth
iTRAQ

ABSTRACT

Tumor size of hepatocellular carcinoma (HCC) is a key parameter for predicting prognosis of HCC patients. The biological behaviors of HCC, such as tumor growth, recurrence and metastasis are significantly associated with tumor size. However, the underlying molecular mechanisms remain unclear. Here, we applied iTRAQ-based proteomic strategy to analyze the proteome differences among small, media, large and huge primary HCC tissues. In brief,88 proteins in small HCC, 69 proteins in media HCC, 118 proteins in large HCC and 215 proteins in huge HCC, were identified by comparing the proteome of cancerous tissues with its corresponding non-cancerous tissues. Further analysis of dysregulated proteins involved in signaling revealed that alteration of ERK1/2 and AKT signaling played important roles in the tumorigenesis or tumor growth in all subtypes. Interestingly, alteration of specific signaling was discovered in small and huge HCC, which might reflect specific molecular mechanisms of tumor growth. Furthermore, the dysregulation degree of a group of proteins has been confirmed to be significantly correlated with the tumor size; these proteins might be potential targets for studying tumor growth of HCC. Overall, we have revealed the molecular signatures of HCC with different tumor sizes, and provided fundamental information for further in-depth study.

Biological significance: In this study, we compared the protein expression profiles among different HCC subtypes, including small HCC, media HCC, large HCC and huge HCC for the first time. The results clearly proved that different molecular alterations and specific signaling pathways were indeed involved in different HCC subtypes, which might explain the different malignancy biological behaviors. In addition, the dysregulation degree of a group of proteins has been confirmed to be significantly correlated with the tumor size. We believe that these findings would help us better understand the underlying molecular mechanisms of the tumorigenesis and development of HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignant cancer and the second most common cause of cancer mortality worldwide [1]. Approximately 400,000 people die from HCC every year in Asian countries, which accounts for 51% of the liver cancer related deaths worldwide [2]. Although surgical resection strategies, which have been proved to be the first choice for HCC treatment, are significantly improved during the past decades, the prognosis of HCC patients is still unsatisfactory [3].

In clinical practice, it has been well established that several tumorous factors, including tumor size, portal vein tumor thrombus (PVTT), tumor lesion number, and differentiation grade of tumor, are important predictors of the prognosis of HCC patients. Among these factors, the tumor size is a crucial factor for staging the HCC patients by either Barcelona Clinic Liver Cancer (BCLC) staging classification system [4] or TNM-based staging system [5], and it is also one of the most important parameters to affect the treatment outcomes of HCC patients, such as successful rate of surgical resection, operative complications, survival, recurrence and metastasis [6–10]. According to the tumor size of patients, HCC could be divided into 4 sub-types clinically, including small HCC (diameter ≤ 3 cm), media HCC (3 cm <diameter ≤ 5 cm), large HCC (5 cm < diameter < 10 cm) and huge HCC (diameter ≥ 10 cm). The biological behaviors and clinical therapeutic outcomes of these different HCC subtypes are extremely different from each other. For

* Corresponding authors at: Xihong Road 312, Fuzhou 350025, Fujian Province, People's Republic of China.
E-mail addresses: xiaoloong.liu@gmail.com (X. Liu), drjingfeng@126.com (J. Liu).

http://dx.doi.org/10.1016/j.jprot.2016.09.008
1874-3919/

example, it has been clearly proved that HCC with larger tumor size was remarkably positively associated with higher degree of capsular invasion [11], and higher incidences of microscopic vascular invasion [7]; Ishii et al. have reported that the tumor size was the only independent risk factor for lung metastasis of HCC by analyzing the prognosis of 293 HCC patients who have underwent surgical resection, and significantly higher incidences of lung metastasis occurred in large or huge HCC patients even if there was no macroscopic vascular invasion [8]. Meanwhile, it has also been reported that the one-year recurrence rate of HCC after radical resection in patients with large or huge HCC is remarkably higher than that in patients with small or media HCC [11–15]. Furthermore, the overall survival rate, or five-year survival rate of patients has been clearly proved to be negatively associated with the tumor size [12,16]. However, the molecular signatures rather than the easily visible size features, as well as the underlying molecular mechanisms of the different biological behaviors of these different HCC subtypes are largely unknown, and need to be further elucidated.

The high-throughput quantitative proteomic strategies are ideal tools for systematically characterizing the overall proteome differences among different disease status, such as revealing the differences in protein expression or protein post-translational modifications that are caused by a particular disease status, which might provide fundamental information for in-depth understanding of complicated diseases such as cancer. Especially, the use of isobaric tags for relative and absolute quantitation (iTRAQ) profiling technology is an ultrasensitive and precise approach to simultaneously quantify and compare the proteome differences up to 8 group samples [17,18]. It has been widely used for investigating the molecular mechanisms of the tumorigenesis, tumor development, and recurrence/metastasis of HCC [19,20], as well as for the screening of diagnostic and prognostic biomarkers of HCC [21–23]. For instance, Huang et al. [23] have quantitatively compared the proteome alterations of huge HCC (diameter is larger than 10 cm) at different recurrence/metastasis stages, and discovered two biomarkers for distinguishing and predicting the early recurrence/metastasis of huge HCC through applying the iTRAQ based strategies; Xing et al. have systematically compared the proteome differences, and revealed the possible mechanism differences between the HCC with a single lesion and HCC with multiple lesions by iTRAQ based quantitative analysis [22].

However, the application of iTRAQ labeling in studying the molecular differences among primary HCC tissues with different tumor sizes has never been reported. Herein, this study aims to carefully investigate the molecular signatures of the different HCC subtypes, including small, media, large and huge HCC, by quantitatively comparing the overall proteome of the primary tumor tissues with its corresponding adjacent noncancerous tissues using iTRAQ coupling with two-dimensional liquid chromatography–tandem mass spectrometry (2D LC–MS/MS).

2. Materials and methods

2.1. Sample collection

Primary HCC tissues with different sizes were collected from 60 patients and were divided into 4 groups according to the tumor size, including small HCC group (SC, n = 15), media HCC group (MC, n = 15), large HCC group (LC, n = 15) and huge HCC group (HC, n = 15). The mean diameter of tumor was 2.2 ± 0.5 cm in the SC group, 3.9 ± 0.5 cm in the MC group, 6.9 ± 1.2 cm in the LC group, and 14.3 ± 3.4 cm in the HC group, respectively. Their corresponding adjacent noncancerous tissues were used as control and were divided into another 4 groups as well (SN represented the adjacent noncancerous tissues of small HCC; MN represented the adjacent noncancerous tissues of media HCC; LN represented the adjacent noncancerous tissues of large HCC; and HN represented adjacent noncancerous tissues of huge HCC). The patient demographic and clinical characteristics were summarized in Table 1. All patients received radical surgery at Mengchao Hepatobiliary Hospital of Fujian Medical University from August 2001 to April 2014. All enrolled patients met the following 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 HCV (Hepatitis C virus) negative; (3) Patient received the standard radical resection [24]: 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, the encapsulation of tumor tissue was intact and the boundary of tumor tissue was distinct; and 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 post-operation; and (5) The patient did not undergo any other intervention or therapies before surgery.

Table 1
Patient demographics and clinical characteristics.

	SC (n = 15)	MC (n = 15)	LC (n = 15)	HC (n = 15)
Gender				
Male	14	12	12	12
Female	1	3	3	3
Age range	34–71	42–67	34–70	38–67
Diameter of tumor	2.2 ± 0.5	3.9 ± 0.5	6.9 ± 1.2	14.3 ± 3.4
AFP[a]				
>10	9	8	12	12
≤10	6	7	0	2
TNM stage				
I, II	15	13	14	14
III, IV	0	2	1	1
Liver cirrhosis				
None	3	2	2	5
Mild/moderate	9	13	13	10
Severe	3	0	0	0

[a] AFP, alpha-fetoprotein, AFP value >10 is positive through the manufacturer's introduction. Two patients in the LC group and one patient in the HC group didn't accept the AFP examination, respectively.

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 using 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

Protein preparation, peptide labeling and 2D LC-MS/MS analysis were performed following the previously published protocols [22] with slight modification. In brief, the tissues from patients were divided into 8 groups as above-mentioned, including SC paired with SN, MC paired with MN, LC paired with LN and HC paired with HN, respectively.

To extract the proteins from tissues, 300 μL of lysis buffer containing 8 M urea, 2% SDS and 1× Protease Inhibitor Cocktail (Roche Ltd. Switzerland) was added into the mixed samples, then followed by tissue homogenization and sonication on ice. After centrifugation at 17, 000g for 10 min at 4 °C, the supernatant was collected and transferred to a fresh tube. The protein concentration of the raw extraction was quantified by BCA assay (Transgene Biotech, China) following the manufacture's protocol. To minimize the individual differences of patients as much as possible, 5 protein extracts with equal amount (60 μg proteins of each) from the same group were pooled together to consist of one sample. In such case, we have 3 repeated protein extracts in total for each