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A structured proteomic approach identifies 14-3-3Sigma as a novel and reliable protein biomarker in panel based differential diagnostics of liver tumors ☆

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ABSTRACT

Hepatocellular carcinoma (HCC) is a major lethal cancer worldwide. Despite sophisticated diagnostic algorithms, the differential diagnosis of small liver nodules still is difficult. While imaging techniques have advanced, adjuvant protein-biomarkers as glypican3 (GPC3), glutamine-synthetase (GS) and heat-shock protein 70 (HSP70) have enhanced diagnostic accuracy. The aim was to further detect useful protein-biomarkers of HCC with a structured systematic approach using differential proteome techniques, bring the results to practical application and compare the diagnostic accuracy of the candidates with the established biomarkers.

After label-free and gel-based proteomics (n = 18 HCC/corresponding non-tumorous liver tissue (NTLT)) biomarker candidates were tested for diagnostic accuracy in immunohistochemical analyses (n = 14 HCC/NTLT). Suitable candidates were further tested for consistency in comparison to known protein-biomarkers in HCC (n = 78), hepatocellular adenoma (n = 25; HCA), focal nodular hyperplasia (n = 28; FNH) and cirrhosis (n = 28).

Of all protein-biomarkers, 14-3-3Sigma (14-3-3S) exhibited the most pronounced up-regulation (58.8×) in proteomics and superior diagnostic accuracy (73.0%) in the differentiation of HCC from non-tumorous hepatocytes also compared to established biomarkers as GPC3 (64.7%) and GS (45.4%). 14-3-3S was part of the best diagnostic three-biomarker panel (GPC3, HSP70, 14-3-3S) for the differentiation of HCC and HCA which is of most important significance. Exclusion of GS and inclusion of 14-3-3S in the panel (>1 marker positive) resulted in a profound increase in specificity (+44.0%) and accuracy (+11.0%) while sensitivity remained stable (96.0%).

14-3-3S is an interesting protein biomarker with the potential to further improve the accuracy of differential diagnostic process of hepatocellular tumors. This article is part of a Special Issue entitled: Medical Proteomics.

Abbreviations: HCC, hepatocellular carcinoma, GPC3, glypican3; GS, glutamine synthetase; HSP70, heat shock protein 70; NTLT, non-tumorous liver tissue; HCA, hepatocellular adenoma; FNH, focal nodular hyperplasia; 14-3-3S, 14-3-3Sigma; 2D-DIGE, two-dimensional difference in gel electrophoresis; CHC, clathrin heavy chain; EZH2, enhancer of zeste homologue 2; TRAP1, tumor necrosis factor type 1 receptor-associated protein 1; MVP, major vault protein; PPA1, inorganic pyrophosphatase 1; CLIC1, chloride intracellular channel protein 1

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1. Introduction

Hepatocellular carcinoma (HCC) is a major lethal cancer associated primarily with chronic viral infection (hepatitis B and C viruses) and chronic non-viral liver disease (alcoholic, dietary, environmental) [1,2]. Whereas 80% of new cases occur in developing countries, the incidence still increases in many industrialized nations like in the United States, Western Europe, and Japan [1,2]. The long term prognosis of HCC is still poor particularly for larger tumors and/or advanced stages when treatment is less effective [2]. Although imaging techniques like ultrasonography or CT/MRI-scans and serum biomarkers as α-fetoprotein (AFP) or des-γ-carboxy prothrombin (DCP) are effectively used in the routine diagnosis, they exhibit relatively low sensitivity for detecting HCC at a small and early stage when curative results are more likely to achieve [1–4]. In situations with non-distinctive (small) liver nodules, diagnostic liver biopsy is often required. However, the histomorphological discrimination between small well-differentiated HCC and its possible precursor lesions such as dysplastic nodules is sometimes challenging, even for experienced pathologists [3,5,6]. Therefore an international consensus group has developed guidelines for the histopathological diagnosis of HCC and the differential diagnosis of well differentiated HCC versus (high-grade) dysplastic nodules (HGDN) [6]. In addition to the proposed histomorphological criteria the use of immunohistochemical tissue biomarkers has become an important diagnostic tool [4,7].

The discovery of new biomarkers in HCC was predominantly driven by genetic profiling at the RNA level. Using oligonucleotide arrays with 12,600 genes Chuma and colleagues identified the transcript for heat shock protein 70 (HSP70) and also the protein as a sensitive marker for HCC [8]. Llovet and colleagues evaluated 55 candidate genes to determine a signature for the diagnosis of early HCC and found a 3-gene set that discriminated HCC with an accuracy of 94% [9]. In this gene set, glypican3 (GPC3) provided the largest diagnostic potential which was also confirmed by GPC3-immunohistochemistry [9]. Previously, glutamine-synthetase (GS) transcript and protein expression levels had been identified to be markedly increased in HCC by screening a complementary DNA library constructed from a human primary liver cancer [10]. The implementation of these three markers (HSP70, GPC3, GS) in the pathological diagnosis increases the diagnostic accuracy for small and well differentiated HCC which has been demonstrated in surgical specimens and liver biopsies and their usage has been recommended by professional associations (EASL, AASLD) [4,7,11,12].

Recently, proteomic-based approaches were employed to identify potential novel biomarkers in HCC using diverse quantification techniques to reveal differences in protein expression. Most studies used the well-established two-dimensional difference in gel electrophoresis (2D-DIGE) technique followed by identification via mass spectrometry (MS). Seimiya and colleagues analyzed HCC and non-tumor tissue from 10 patients by 2D-DIGE technique and identified 83 differentially expressed proteins by MS [13]. The immunohistochemical validation of the protein Clathrin Heavy Chain (CHC) was demonstrated to be a useful positive marker in the diagnosis of early HCC [3,13]. By using a similar study design Cai and colleagues found the protein enhancer of Zeste Homologue 2 (EZH2) useful as a HCC marker [14].

Although 2D-DIGE is an accurate and sensitive technique, the requirement of a large amount of protein and associated high costs of labeling reagents are considerable disadvantages. Label-free proteomic approaches utilizing spectral counting or ion intensity-based quantification offer an alternative proteomic-based biomarker discovery method [15,16]. These methods are cheaper due to the lacking need of labeling reagents and allow for high-throughput and sensitive analyses in a mass spectrometer.

In a recent study we applied the two different techniques of 2D-DIGE and a label-free ion intensity-based quantification via MS and liquid chromatography to identify differentially expressed proteins in HCC and the corresponding non-tumorous liver tissue (NTLT) [16]. In the present study we combined these results with an additional label-free proteome analysis using a larger patient cohort to detect biomarkers for HCC. The aim of the present study was to transfer the results of our systematic high-throughput proteomic approach to practical immunohistochemical application and to compare the diagnostic accuracy of our candidates with the established diagnostic biomarkers HSP70, GPC3 and GS and with the recently described biomarkers CHC and EZH2 [3,11,12,14].

2. Materials and methods

The study design is shown in the flow chart diagram including the sequence of conducted analyses (Fig. 1).

2.1. Biomarker detection set

Tissue samples of HCC and concomitant NTLT were collected from patients after resection or liver transplantation for HCC. Tumor and NTLT samples were snap frozen and stored at −80 °C for proteomic analyses and western blotting. Also, tumorous and NTLT samples were fixed in 4% buffered formalin and paraffin embedded (FFPE). Slides were prepared and stained with hematoxylin and eosin (HE) according to institutional standards. The tumors were diagnosed according to current WHO-criteria and classified according to the TNM-system (7th edition) [17,18].

The study protocol was in conformance with the ethical guidelines of the 1975 Declaration of Helsinki as revised in 1983. The local ethics committee approved the study (11-4839-BO).

2.1.1. Differential proteome analyses

The current label-free proteomic study was performed in analogy to our previously published one [16], but an enlarged and mostly independent patient cohort (overlap 2/18) was used with a total of 22 samples (n = 11 HCC, n = 11 NTLT) and the analytical workflow was slightly modified in accordance to a recently published procedure [19]. In detail, frozen tissue samples were lysed in sample buffer (30 mM Tris HCl; 2 M thiourea, 7 M urea, pH 8.5) and protein concentration was determined using Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). A protein amount of 10 μg of each sample was concentrated via 1D SDS short gel (samples were allowed to run approx. 1 cm into the gel). After Coomassie staining, gel bands were excised and subjected to tryptic protein digestion for 16 h at 37 °C (1:50 enzyme to protein ratio). Tryptic peptides were extracted twice with 20 μl of 50% acetonitrile in 0.1% trifluoroacetic acid (TFA). Combined extracts were dried in vacuo and resolved in 0.1% TFA and peptide concentration was subsequently determined using amino acid analysis. An amount of 350 ng of each peptide sample was then analyzed by LC–MS/MS (Ultimate 3000 RSLCnano system online coupled to a Orbitrap Elite instrument (both Thermo Scientific, Bremen, Germany). Initially, peptides were trapped on a pre-column (Acclaim® PepMap 100, 75 μm × 2 cm, nano Viper, C18, 2 μm, 100 Å) over 10 min with 0.1% TFA at a flow rate of 7 μl/min. Then, peptide separation was performed on an analytical column (Acclaim® PepMap RSLC, 75 μm × 50 cm, nano Viper, C18, 2 μm, 100 Å) using a 90 min gradient at 60 °C 5 to 40% solvent B at a flow rate of 300 nl/min (solvent A: 0.1% formic acid; solvent B: 0.1% formic acid in 84% acetonitrile). The mass spectrometer was operated in a data-dependent mode. Full scan MS spectra (mass range 350–2000 m/z) were acquired in the Orbitrap analyzer at a mass resolution of 60,000. Twenty most-abundant ions per spectra were then fragmented using collision-induced dissociation (35% normalized collision energy) and scanned in the linear ion trap. Protein and peptide identifications were performed using Proteome Discoverer software (ver. 1.3.0.339, Thermo Fisher Scientific, Rockford IL, USA) and Mascot search algorithm (ver. 2.3.0.2) (Matrix Science Ltd., London, UK) was used for searching against UniProtKB/Swiss-Prot database (release