Pathology (October 2015) **47(6)**, pp. 543–550

ANATOMICAL PATHOLOGY

Differential proteomic and tissue expression analyses identify valuable diagnostic biomarkers of hepatocellular differentiation and hepatoid adenocarcinomas

Henning Reis[1], Juliet Padden[2], Maike Ahrens[2], Carolin Pütter[3], Stefanie Bertram[1], Leona L. Pott[1,2], Anna-Carinna Reis[1,4], Frank Weber[5], Benjamin Juntermanns[5], Andreas-C. Hoffmann[6], Martin Eisenacher[2], Joörg F. Schlaak[7], Ali Canbay[7], Helmut E. Meyer[2,8], Barbara Sitek[2,*] and Hideo A. Baba[1,*]

[1]Institute of Pathology, University Hospital of Essen, University of Duisburg-Essen, Essen, [2]Medizinisches Proteom-Center, Ruhr-Universität Bochum, Bochum, [3]Institute for Medical Informatics, Biometry and Epidemiology, University Hospital of Essen, University of Duisburg-Essen, Essen, [4]Institute of Pathology, Klinikum Dortmund gGmbH, Dortmund, [5]Department of General, Visceral and Transplantation Surgery, University Hospital of Essen, University of Duisburg-Essen, Essen, [6]West German Cancer Centre Essen, University Hospital of Essen, University of Duisburg-Essen, Essen, [7]Department of Gastroenterology and Hepatology, University Hospital of Essen, University of Duisburg-Essen, Essen, and [8]Leibniz-Institut für Analytische Wissenschaften - ISAS - e.V., Dortmund, Germany; [*]contributed equally as senior authors.

Summary

The exact discrimination of lesions with true hepatocellular differentiation from secondary tumours and neoplasms with hepatocellular histomorphology like hepatoid adenocarcinomas (HAC) is crucial. Therefore, we aimed to identify ancillary protein biomarkers by using complementary proteomic techniques (2D-DIGE, label-free MS). The identified candidates were immunohistochemically validated in 14 paired samples of hepatocellular carcinoma (HCC) and non-tumourous liver tissue (NT). The candidates and HepPar1/Arginase1 were afterwards tested for consistency in a large cohort of hepatocellular lesions and NT ($n=290$), non-hepatocellular malignancies ($n=383$) and HAC ($n=13$). Eight non-redundant, differentially expressed proteins were suitable for further immunohistochemical validation and four (ABAT, BHMT, FABP1, HAOX1) for further evaluation. Sensitivity and specificity rates for HCC/HAC were as follows: HepPar1 80.2%, 94.3% / 80.2%, 46.2%; Arginase1 82%, 99.4% / 82%, 69.2%; BHMT 61.4%, 93.8% / 61.4%, 100%; ABAT 84.4%, 33.7% / 84.4%, 30.8%; FABP1 87.2%, 95% / 87.2%, 69.2%; HAOX1 95.5%, 36.3% / 95.5%, 46.2%. The best 2×/3× biomarker panels for the diagnosis of HCC consisted of Arginase1/HAOX1 and BHMT/Arginase1/HAOX1 and for HAC consisted of Arginase1/FABP1 and BHMT/Arginase1/FABP1. In summary, we successfully identified, validated and benchmarked protein biomarker candidates of hepatocellular differentiation. BHMT in particular exhibited superior diagnostic characteristics in hepatocellular lesions and specifically in HAC. BHMT is therefore a promising (panel based) biomarker candidate in the differential diagnostic process of lesions with hepatocellular aspect.

Key words: Arginase1, BHMT, diagnosis, hepatocellular carcinoma, hepatoid adenocarcinoma, HepPar1, immunohistochemistry.

Received 24 February 2015, accepted 19 April 2015

INTRODUCTION

Hepatocellular carcinoma (HCC) is a major lethal cancer worldwide.[1] As the histopathological discrimination of hepatocellular liver tumours can be challenging, especially of early well differentiated HCC and dysplastic nodules, immunohistochemical protein biomarkers have become an important diagnostic tool.[2–4] Apart from considerations in tumours obviously arising in and from the liver, histopathological resemblance to hepatocytes can occur in a variety of carcinomas at different sites ('hepatoid adenocarcinomas', HAC).[5–7] Additionally, a hepatocellular origin is often assumed in metastatic adenocarcinomas initially diagnosed as carcinomas of unknown primary (CUP) but being metastases of a primary HCC.[8] To facilitate the differential diagnostic process and therefore enable a timely and adequate therapy, diagnostic protein biomarkers of hepatocellular differentiation are needed.

An immunohistochemical differential diagnostic approach in an adenocarcinoma suspected of or exhibiting features of a HCC usually includes staining of cytokeratin 7 and 20 (CK7, CK20), both of which are negative in the vast majority of HCCs.[9,10] A further step can include antibodies such as Glypican 3 (GPC), alpha-fetoprotein (AFP) and polyclonal carcinoembryogenic antigen (CEA) but will most probably include HepPar1 (hepatocyte paraffin 1) designated as the 'classic' marker of hepatocellular differentiation.[9] In 1993, HepPar1 was developed as a mouse monoclonal antibody with formalin fixed liver tissue as the immunogenic antigen.[11] Not until 2008 was the target of HepPar1's immunoreactivity identified as carbamoyl phosphate synthetase 1, a mitochondrial enzyme linked to the production of urea.[12] HepPar1 is expressed in non-tumourous hepatocytes and usually exhibits high positivity rates in HCC with a range reported from 69% to 100%.[13] However, HepPar1 only reacts in a low percentage of poorly differentiated HCC (14–57%) and scirrhous HCC (<50%). Additionally, HepPar1 immunoreactivity has been described in a variety of non-hepatocellular tumours such as adenocarcinomas of the

Print ISSN 0031-3025/Online ISSN 1465-3931
DOI: 10.1097/PAT.0000000000000298

lung and gallbladder, cholangiocellular and adrenal cortical carcinomas. The highest rates of HepPar1 reactivity were detected in adenocarcinomas with hepatoid morphology of the gastro-intestinal tract and pancreas as well as yolk sac tumours, thus constituting a particular diagnostic challenge.[9,13]

In recent years, evidence aggregates that another enzyme involved in the urea cycle, Arginase1, is an additional valuable protein biomarker of hepatocellular differentiation. Originally described in 2010,[14] Arginase1 was found to be highly expressed in non-tumourous hepatocytes as well as in hepatic adenomas, focal nodular hyperplasias (FNH) and dysplastic nodules.[14] The sensitivity of Arginase1 was reported to be 79–96% in HCC, with the lowest rates in poorly differentiated HCC (43%) which is still a 29% higher rate compared to HepPar1.[13] Additionally, 85% of scirrhous HCC were found to express Arginase1 which is profoundly higher than HepPar1's immunopositivity rate (37.5%). However, Arginase1 was found, albeit to a much lower degree than HepPar1, to be expressed in non-hepatic tumours, for example in adenocarcinomas of the pancreas.[13]

As a result, new diagnostic protein biomarkers of hepatocellular differentiation are needed to further enhance the differential diagnostic process of tumours with hepatocellular morphology or origin. Therefore, we selected potential protein biomarkers of hepatocellular differentiation, identified in a structured proteomic approach using differential proteomic techniques,[15] to validate these candidates in a large cohort of benign liver tumours and HCC as well as in non-liver tumours. The candidates were compared to known biomarkers of hepatocellular differentiation. Additionally, the best diagnostic biomarker panels were calculated and the diagnostic performance was evaluated with emphasis on HAC (Fig. 1).

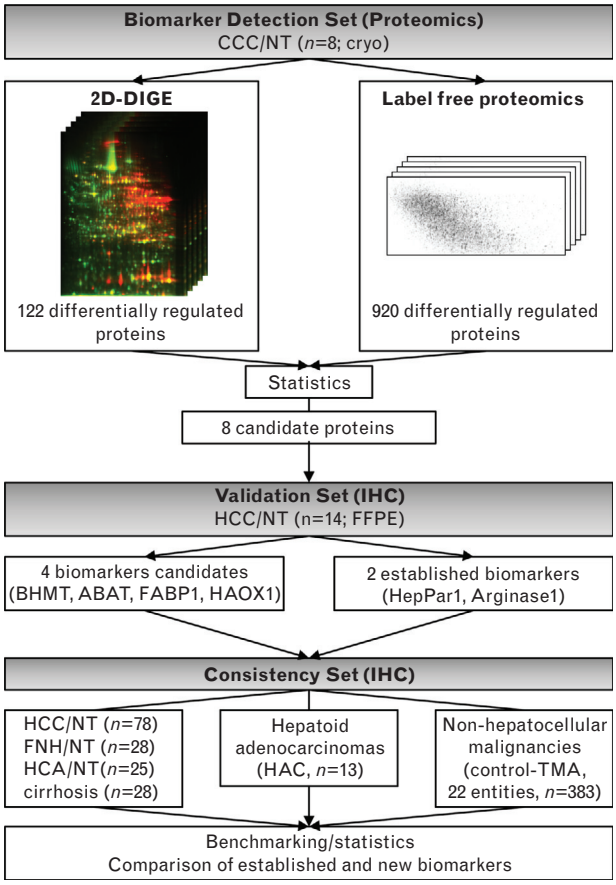

Fig. 1 Schematic workflow diagram. Protein biomarker candidates were detected in a structured proteomic approach (Biomarker Detection Set[15]) and further tested in hepatocellular carcinoma (HCC) and non-tumourous liver tissue (NT) by immunohistochemistry (Validation Set). The biomarker candidates and established biomarkers were further benchmarked in the large cohorts of hepatocellular tumours and NT ($n = 290$), hepatoid adenocarcinomas ($n = 13$) and non-hepatocellular malignancies ($n = 383$) (Consistency Set). CCC, cholangiocellular carcinoma; NT, non-tumourous liver tissue; 2D-DIGE, two-dimensional difference in gel electrophoresis; IHC, immunohistochemistry; HCC, hepatocellular carcinoma; FFPE, formalin fixed and paraffin embedded; FNH, focal nodular hyperplasia; HCA, hepatocellular adenoma; TMA, tissue microarray.

MATERIALS AND METHODS

Proteomic analyses (biomarker detection set)

The schematic workflow is depicted in Fig. 1.

In the previous proteomic study, we compared cholangiocellular carcinoma (CCC) tissue with non-tumourous liver tissue (NT) from the same patients ($n = 8$).[15] Besides promising biomarker candidates up-regulated in CCC tissue, we also detected proteins down-regulated in CCC tissue. Our hypothesis for the current study was these biomarkers being specific for hepatocytes, i.e., hepatocellular differentiation. Accordingly, candidate proteins with higher abundance in NT were chosen for further evaluation.

Detailed information on this sample set and proteomic techniques have been published previously.[15] In brief, CCC and corresponding NT tissues were collected from eight patients, snap frozen and stored at −80°C until further processing. Proteins were extracted and analysed by two-dimensional difference in-gel electrophoresis (2D-DIGE) and label-free liquid chromatography tandem-mass spectrometry (LC-MS/MS).

For 2D-DIGE, proteins were labelled with cyanine dyes (GE Healthcare, Germany) and separated by isoelectric focusing and second-dimension sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in self-made gels. Gel images were acquired on a Typhoon 9400 (Amersham Biosciences, GE Healthcare, UK), preprocessed using Image Quant (GE Healthcare) and evaluated for differential protein spots with DeCyder2D Software (GE Healthcare). Here, the following criteria had to be met: (1) protein spots had to be present in at least 70% of all spot maps, (2) the p value of Student's t-test (paired, two-sided) had to be ≤ 0.05 (after adjustment for multiple testing, controlling the false discovery rate using the method of Benjamini and Hochberg), and (3) the average ratio between experimental groups had to be at least 1.5. Identification of differentially regulated proteins was achieved by matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) on an Ultra-Flex II (Bruker Daltronics, Germany).

Label-free analysis was performed, after in-gel digestion of tissue lysate with trypsin, on an Ultimate 3000 RSLCnano system (Dionex, Germany) online coupled to an LTQ Orbitrap Elite (Thermo Scientific, USA). Progenesis LC-MS software (v. 4.0.4265.42984; Nonlinear Dynamics, UK) was used for ion-intensity-based quantification and proteins were identified by Proteome Discoverer (v. 1.3; Thermo Scientific) searching the UniProt database (release 2012 02, 534,695 entries) via Mascot (v.2.3.0.2; Matrix Sciences, UK). Again, only proteins with a p value of Students t-test (paired, two-sided) ≤ 0.05 after false discovery rate correction and an absolute fold change ≥ 1.5 were considered to be differentially regulated.

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and the local ethics committee of the Medical Faculty of the University of Duisburg-Essen approved the study. Written informed consent was obtained from the patients. Clinicopathological parameters of the cohort are displayed in Table 1.

Immunohistochemical (IHC) validation (validation set)

Suitable candidates from proteome analyses were further technically validated in a test cohort of HCC (validation set). Therefore, a tissue microarray (TMA) block of formalin fixed, paraffin embedded (FFPE) tissue was constructed including matched HCC and NT from 14 patients (total $n = 28$) with three cores in each case (core diameter 1 mm). All reactions were conducted on 1–2 μm thick tissue slides on an automated staining device (Dako Autostainer; Dako, Denmark) after dewaxing and pre-treatment. A control was included in every