Journal of Proteomics 154 (2017) 13–21

Contents lists available at ScienceDirect

Journal of Proteomics

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

One Sample, One Shot - Evaluation of sample preparation protocols for the mass spectrometric proteome analysis of human bile fluid without extensive fractionation

Dominik A. Megger [a,*], Juliet Padden [a], Kristin Rosowski [a], Julian Uszkoreit [a], Thilo Bracht [a], Martin Eisenacher [a], Christian Gerges [b], Horst Neuhaus [b], Brigitte Schumacher [c], Jörg. F. Schlaak [d], Barbara Sitek [a]

[a] Medizinisches Proteom-Center, Ruhr-Universität Bochum, Germany
[b] Department of Internal Medicine, Evangelisches Krankenhaus, Düsseldorf, Germany
[c] Klinik für Innere Medizin und Gastroenterologie, Elisabeth Krankenhaus Essen, Essen, Germany
[d] Department of Internal Medicine, Evangelisches Krankenhaus Duisburg-Nord, Duisburg, Germany

ARTICLE INFO

Article history:
Received 18 August 2016
Received in revised form 8 November 2016
Accepted 29 November 2016
Available online 05 December 2016

Keywords:
Bile fluid
proteomics
method comparison
biomarkers
hepatocellular carcinoma
cholangiocellular carcinoma

ABSTRACT

The proteome analysis of bile fluid represents a promising strategy to identify biomarker candidates for various diseases of the hepatobiliary system. However, to obtain substantive results in biomarker discovery studies large patient cohorts necessarily need to be analyzed. Consequently, this would lead to an unmanageable number of samples to be analyzed if sample preparation protocols with extensive fractionation methods are applied. Hence, the performance of simple workflows allowing for "one sample, one shot" experiments have been evaluated in this study. In detail, sixteen different protocols implying modifications at the stages of desalting, delipidation, deglycosylation and tryptic digestion have been examined. Each method has been individually evaluated regarding various performance criteria and comparative analyses have been conducted to uncover possible complementarities. Here, the best performance in terms of proteome coverage has been assessed for a combination of acetone precipitation with in-gel digestion. Finally, a mapping of all obtained protein identifications with putative biomarkers for hepatocellular carcinoma (HCC) and cholangiocellular carcinoma (CCC) revealed several proteins easily detectable in bile fluid. These results can build the basis for future studies with large and well-defined patient cohorts in a more disease-related context.

Biological significance: Human bile fluid is a proximal body fluid and supposed to be a potential source of disease markers. However, due to its biochemical composition, the proteome analysis of bile fluid still represents a challenging task and is therefore mostly conducted using extensive fractionation procedures. This in turn leads to a high number of mass spectrometric measurements for one biological sample. Considering the fact that in order to overcome the biological variability a high number of biological samples needs to be analyzed in biomarker discovery studies, this leads to the dilemma of an unmanageable number of necessary MS-based analyses. Hence, easy sample preparation protocols are demanded representing a compromise between proteome coverage and simplicity.

In the presented study, such protocols have been evaluated regarding various technical criteria (e.g. identification rates, missed cleavages, chromatographic separation) uncovering the strengths and weaknesses of various methods. Furthermore, a cumulative bile proteome list has been generated that extends the current bile proteome catalog by 248 proteins. Finally, a mapping with putative biomarkers for hepatocellular carcinoma (HCC) and cholangiocellular carcinoma (CCC) derived from tissue-based studies, revealed several of these proteins being easily and reproducibly detectable in human bile. Therefore, the presented technical work represents a solid base for future disease-related studies.

1. Introduction

In the field of biomarker research, body fluids are of high interest as they are supposed to be potential sources of biomarkers for various diseases, in particular cancer. Especially plasma and serum are of high interest because both can be routinely collected in a minimal-invasive

* Corresponding author at: Medizinisches Proteom-Center, Ruhr-Universität Bochum, Universitätsstr. 150, 44801 Bochum, Germany.
E-mail address: dominik.megger@rub.de (D.A. Megger).

http://dx.doi.org/10.1016/j.jprot.2016.11.021
1874-3919/

manner. However, due to the fact that only a few high-abundant proteins account for the largest part of total protein content in serum and plasma, changes in the low-abundant region are mostly hard to detect or completely masked, especially if MS-based proteomics approaches without depletion of high-abundant proteins are applied. In contrast to this, the analysis of proximal body fluids derived from the close vicinity of a particular disease seems to be a more promising strategy for proteomics-based biomarker discovery and validation [1].

In the case of diseases of the hepatobiliary system, bile fluid represents one of the proximal body fluids of interest as it is produced by hepatocytes and transported through the intrahepatic bile ducts to the gallbladder. There it is stored and concentrated until its release through the extrahepatic bile duct into the duodenum [2]. Due to this flow through the hepatobiliary tract, the probability to detect changes of protein abundances associated with a particular disease in this region is necessarily higher in bile than in serum or plasma. This advantage compensates the drawback that in comparison to the collection of blood bile fluid needs to be collected via comparatively more invasive methods such as endoscopic retrograde cholangiopancreatography (ERCP). However, based on its biochemical composition, the proteome analysis of bile fluid still remains a challenging task as proteins account only for a small part with a total concentration of 1 - 8 μg/μl [2–3]. Instead, the main bile components are bile salts, bilirubin, cholesterol, fatty acids, lecithins and electrolytes [2,4].

Several studies reporting proteome analyses of human bile using high-resolution mass spectrometry have been published in the past few years, steadily increasing the knowledge about the proteome composition of this particular body fluid. For example, Farina et al. applied fractionation of bile fluid based on stepwise centrifugation and protein separation via SDS-PAGE. With a protein digestion procedure combining deglycosylation and tryptic digestion a total of 812 unique protein identifications was achieved [5]. In the same year, Farid and co-workers applied depletion of albumin and immunoglobulin G in combination with extensive SDS-PAGE fractionation to yield a comparable number of 813 unique proteins identified [6]. Again in 2011, Barbhuiya et al. reported the most comprehensive bile proteome list with 2,552 unique identifications using a combination of various sample preparation steps, namely lipid removal, desalting, protein depletion, SCX fractionation, SDS-PAGE, and OFFGEL fractionation [7]. Based on these three and other studies a comprehensive picture of the bile proteome consisting of 3,099 different gene products has been drawn [2]. Whereas each of these studies gave valuable and important results according the composition of the bile proteome, in all cases extensive sample preparation procedures containing multiple steps were applied. Especially, the implied analysis of multiple SDS-PAGE fractions makes these protocols not applicable for high-throughput analysis of large patient cohorts and subsequent quantitative proteome analysis using label-free approaches. As here each sample is processed independently from sample acquisition to final LC-MS/MS measurement, the analysis of multiple fractions of a given sample would result in a tremendously increased sample number [8]. Hence, protocols enabling "one sample, one shot" analyses of human bile fluid are demanded.

In the reported study, we aimed for a systematic evaluation of such comparatively simple sample preparation protocols of human bile that imply a compromise between proteome coverage and simplicity, thereby principally allowing for quantitative analyses of large sample numbers in a label-free manner. The respective experimental workflow of the study is shown in Fig. 1. By combining different methods for desalting, delipidation, deglycosylation and tryptic digestion, sixteen different protocols were tested in total. Based on the results of three to four replicate experiments, the performance of each protocol was assessed and direct comparisons between protocols were conducted to uncover potential complementarities. Furthermore, identified bile proteins were functionally annotated and compared with published lists of putative HCC and CCC biomarker candidates [9–11] to reveal proteins that are principally detectable in bile without tedious fractionation procedures, and therefore ideal candidates for future high-throughput validation experiments in a disease-related context.

2. Material and methods

2.1. Patient information and sample acquisition

Within this study a pool of bile samples collected from 18 patients was analyzed. All samples were obtained during ERCP. The corresponding local ethical committees had approved sample collections (Nr.: 11-4797-BO). A table with patient characteristics is attached as supporting information.

2.2. General sample preparation

The pooled bile sample containing equal volumes of each individual sample was diluted with DPBS (-/-)(PAN-Biotech, Aidenbach, Germany) at a ratio of 1:4. Afterwards, the diluted bile fluid was centrifuged for 10 min at 4,800 x g and 4 °C. The protein concentration (0.4 μg/μl) of the supernatant was determined by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). Aliquots of 1.5 ml were prepared and stored at -80 °C until further analysis.

2.3. Delipidation

For delipidation, the Cleanascite™ Lipid Removal Reagent and Clarification Kit (BSG, NJ 08852, USA) was used following manufacturer's instructions. Briefly, Cleanascite™ reagent and sample were mixed at a 1:4 ratio. The mixture was shaken gently for 10 min at room temperature and centrifuged for 1 min at 16,100 x g. The resulting supernatant was used for further experimental steps. Protein quantification of the supernatant revealed an overall recovery of protein sample of 40 - 50 %.

2.4. Acetone precipitation

For protein precipitation 1.2 ml pre-chilled acetone was added to 300 μl of sample and the mixture was stored over night at -20 °C. The resulting suspension was centrifuged for 15 min at 16,100 x g at 4 °C. After removing the supernatant, the pellet was air dried for 5 min. Depending on the following experimental steps, the precipitate was resuspended in 200 μl 50 mM ammonium bicarbonate buffer (no subsequent deglycosylation) or 200 μl 50 mM sodium phosphate buffer (subsequent deglycosylation). Irrespective of the applied solubilization buffer, a protein recovery of 80 - 90 % was assessed using BCA assay.

2.5. Buffer exchange

Amicon® Ultra 10K 0.5 ml centrifugal filters for DNA and protein purification and concentration (Merck Millipore, Darmstadt, Germany) were used for buffer exchange. Following manufacturer's instructions, 500 μl of bile fluid sample was concentrated to a volume of 15 μl and rebuffered in 100 μl of 50 mM ammonium bicarbonate buffer (no subsequent deglycosylation) or 50 mM sodium phosphate buffer (subsequent deglycosylation) depending on the following experimental steps.

2.6. Deglycosylation

Protein Deglycosylation Mix (New England Biolabs GmbH, Frankfurt am Main, Germany) was used for deglycosylation. Depending on the subsequent digestion procedure, slightly different protocols were followed. In the case of in-solution digestion, 5 μl of deglycosylation enzyme cocktail were added to the sample after the alkylation step (see 2.7), followed by an incubation time of 4 h at 37 °C. In the case of in-gel digestion, an amount of 100 μg protein sample (40 μl) was mixed with 5 μl of Deglycosylation Enzyme Cocktail and incubated for 4 h at 37 °C. Afterwards, the samples were prepared for subsequent in-gel