



LIQUID BIOPSY PROTEINS AS PSC-SPECIFIC AND PAN-CCA BIOMARKERS OF CANCER RISK, EARLY DIAGNOSIS AND SURVIVAL MIRRORING TUMOR CELLS

Ainhoa Lapitz¹, Mikel Azkargorta^{2,3}, Piotr Milkiewicz^{4,5}, Paula Olaizola¹, Ekaterina Zhuravleva⁶, Marit M. Grimsrud⁷, Christoph Schramm^{8,9,10}, Ander Arbelaiz¹, Colm J. O'Rourke⁶, Adelaida La Casta¹, Malgorzata Milkiewicz¹², Tania Pastor¹, Mette Vesterhus^{7,11}, Raul Jiménez-Agüero¹, Michael T. Dill^{13,14}, Angela Lamarca¹⁵, Juan W. Valle¹⁵, Rocio I.R. Macias^{3,16}, Laura Izquierdo-Sánchez¹, Ylenia Pérez Castaño^{1,17}, Francisco Javier Caballero-Camino¹, Ioana Riano¹, Marcin Krawczyk^{18,19}, Cesar Ibarra²⁰, Javier Bustamante²⁰, Luiz Miguel Nova-Camacho²¹, Juan M. Falcon-Pérez^{3,22,23}, Felix Elortza^{2,3}, Maria J. Perugorria^{1,3,24}, Jesper B. Andersen⁶, Luis Bujanda^{1,3}, Tom H. Karlsen⁷, Trine Folseraas^{7,25}, Pedro M. Rodrigues^{1,3,23} and Jesus M. Banales^{1,3,23,26}

¹Department of Liver and Gastrointestinal Diseases, Biodonostia Health Research Institute-Donostia University Hospital, University of the Basque Country (UPV/EHU), San Sebastian. ²Proteomics Platform, CIC bioGUNE, Basque Research and Technology Alliance (BRTA), ProteoRed-ISCIII, Bizkaia Science and Technology Park, Derio. ³National Institute for the Study of Liver and Gastrointestinal Diseases (CIBERehd), ISCIII, Madrid. ⁴Liver and Internal Medicine Unit, Department of General, Transplant and Liver Surgery, Medical University of Warsaw, Poland. ⁵Translational Medicine Group, Pomeranian Medical University, Szczecin, Poland. ⁶Biotech Research and Innovation Centre, Department of Health and Medical Sciences, University of Copenhagen, Denmark. ⁷Norwegian PSC Research Center, Department of Transplantation Medicine, Division of Surgery, Inflammatory Medicine and Transplantation, Oslo University Hospital, Rikshospitalet, Oslo, Norway. ⁸European Reference Network Hepatological Diseases (ERN RARE-LIVER), Hamburg, Germany. ⁹1st Department of Internal Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany. ¹⁰Martin Zeitz Centre for Rare Diseases, University Medical Centre Hamburg-Eppendorf, Hamburg, Germany. ¹¹Department of Clinical Science, University of Bergen, Bergen, Norway. ¹²Department of Medical Biology, Pomeranian Medical University in Szczecin, Poland. ¹³Department of Gastroenterology, Infectious Diseases and Intoxication, Heidelberg University Hospital, Heidelberg, Germany. ¹⁴Experimental Hepatology, Inflammation and Cancer, German Cancer Research Center (DKFZ), Heidelberg, Germany. ¹⁵Department of Medical Oncology, The Christie NHS Foundation Trust/Division of Cancer Sciences, University of Manchester, Manchester, UK. ¹⁶Experimental Hepatology and Drug Targeting (HEVEPHARM), University of Salamanca, Biomedical Research Institute of Salamanca (IBSAL), Salamanca. ¹⁷Osakidetza Basque Health Service, Bidasoa IHO, Bidasoa Hospital, Department of Digestive System, Irun. ¹⁸Department of Medicine II, Saarland University Medical Centre, Saarland University, Homburg, Germany. ¹⁹Laboratory of Metabolic Liver Diseases, Centre for Preclinical Research, Department of General, Transplant and Liver Surgery, Warsaw, Poland. ²⁰Osakidetza Basque Health Service, Ezkerraldea-Enkarterri-Cruces IHO, Cruces University Hospital, Barakaldo. ²¹Osakidetza Basque Health Service, Donostialdea IHO, Donostia University Hospital, Department of Pathology, San Sebastian. ²²Center for Cooperative Research in Biosciences (CIC bioGUNE), Basque Research and Technology Alliance (BRTA), Exosomes Laboratory, Derio. ²³Ikerbasque, Basque Foundation for Science, Bilbao. ²⁴Department of Medicine, Faculty of Medicine and Nursing, University of the Basque Country, UPV/EHU, Leioa. ²⁵Section of Gastroenterology, Department of Transplantation Medicine, Oslo University Hospital, Oslo, Norway. ²⁶Department of Biochemistry and Genetics, School of Sciences, University of Navarra, Pamplona.

Resumen

Introduction and objectives: Cholangiocarcinomas (CCAs), heterogeneous biliary tumors with

dismal prognosis, lack accurate early-diagnostic methods, especially important for individuals at high-risk (i.e., primary sclerosing cholangitis (PSC)). Here, we searched for protein biomarkers in serum extracellular vesicles (EVs).

Methods: EVs from patients with isolated PSC (n = 45), concomitant PSC-CCA (n = 42), PSC who developed CCA during follow-up (PSC to CCA; n = 25), CCAs from non-PSC etiology (n = 56), hepatocellular carcinoma (n = 34) and healthy individuals (n = 55) were characterized by mass-spectrometry. Diagnostic biomarkers of PSC-CCA, non-PSC CCA or CCAs regardless etiology (pan-CCAs) were defined, and their expression was evaluated in human organs/tissues and within CCA tumors at single-cell level. Prognostic EV-biomarkers for CCA were investigated.

Results: High-throughput proteomics identified candidate diagnostic biomarkers for PSC-CCA, non-PSC CCA or pan-CCA, as well as and for differential diagnosis of intrahepatic CCA and HCC, that were cross-validated by ELISA using total serum. Machine learning logit modelling disclosed CRP/FRIL/Fibrinogen algorithm with diagnostic value for early-stage PSC-CCA v s isolated PSC (AUC = 0.944; OR = 82.0), overpowering CA19-9 (AUC = 0.735; OR = 9.3). An algorithm combining CRP/VWF/PIGR//Fibrinogen allowed the diagnosis of early-stage non-PSC CCAs compared to healthy individuals (AUC = 0.999; OR = 1,115). Noteworthy, levels of Fibrinogen/CRP/PIGR/FRIL showed predictive capacity for CCA development in patients with PSC before clinical evidences of malignancy. Multi-organ transcriptomic analysis revealed that serum EVbiomarkers were mostly expressed in hepatobiliary tissues, and scRNA-seq and immunofluorescence analysis of CCA tumors showed their presence mainly in malignant cholangiocytes. Multivariable analysis unveiled EV-prognostic biomarkers independent to clinical features, with COMP/GNAI2/CFAI and ACTN1/MYCT1/PF4V associated negatively or positively to patients' survival, respectively.

Conclusions: Serum EVs contain protein biomarkers for the prediction, early diagnosis and prognosis estimation of CCA, representing a novel tumor cell-derived liquid biopsy for personalized medicine.