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Metastatic Tissue Proteomic Profiling Predicts 5-Year Outcomes in Patients with Colorectal Liver Metastases[1]

Santiago Marfà*, Josep Marti†, Adalgiza Reyes†, Gregori Casals*, Guillermo Fernández-Varo*,‡, Silvia Carvajal*, J.C. García-Valdecasas†, Josep Fuster† and Wladimiro Jiménez*,‡

*Biochemistry and Molecular Genetics Service, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBEREHD), Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain; †HepatoBilioPancreatic Surgery and Transplant Unit Department of Surgery, ICMDM, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBEREHD), Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), University of Barcelona, Barcelona, Spain; ‡Department of Biomedicine, University of Barcelona, Barcelona, Spain

Abstract

Colorectal cancer (CRC) is one of the most common cancers in the developed countries, and nearly 70% of patients with CRC develop colorectal liver metastases (CRLMs). During the last decades, several scores have been proposed to predict recurrence after CRLM resection. However, these risk scoring systems do not accurately reflect the prognosis of these patients. Therefore, this investigation was designed to identify a proteomic profile in human hepatic tumor samples to classify patients with CRLM as "mild" or "severe" based on the 5-year survival. The study was performed on 85 CRLM tumor samples. Firstly, to evaluate any distinct tumor proteomic signatures between mild and severe CRLM patients, a training group of 57 CRLM tumor samples was characterized by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry, and a classification and regression tree (CART) analysis was subsequently performed. Finally, 28 CRLM tumor samples were used to confirm and validate the results obtained. Based on all the protein peaks detected in the training group, the CART analysis was generated, and four peaks were considered to be the most relevant to construct a diagnostic algorithm. Indeed, the multivariate model yielded a sensitivity of 85.7% and a specificity of 86.1%, respectively. In addition, the receiver operating characteristic (ROC) curve showed an excellent diagnostic accuracy to discriminate mild from severe CRLM patients (area under the ROC: 0.903). Finally, the validation process yielded a sensitivity and specificity of 68.8% and 83.3%, respectively. We identified a proteomic profile potentially useful to determine the prognosis of CRLM patients based on the 5-year survival.

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Address all correspondence to: Dr. Wladimiro Jiménez, Servicio de Bioquímica y Genética Molecular, Hospital Clinic Universitari, Villarroel 170, Barcelona 08036, Spain.
E-mail: wjimenez@clinic.ub.es

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Introduction

Colorectal cancer (CRC) is the third most common cancer in the United States in terms of incidence and mortality [1], and similar results are also observed in other developed countries [2]. Nearly 70% of patients with CRC develop colorectal liver metastases (CRLMs), and 10% to 25% are diagnosed at the time of resection of the primary tumor [3,4]. At present, surgical resection still remains the most effective procedure to lengthen patient survival and is the only curative treatment [5,6], with a 5-year survival rate of CRLM after hepatic resection ranging from 30% to 60% [7]. Although some patients are not surgical candidates, recent advances in chemotherapy, radiofrequency ablation, and thermoablative methods have increased the number of patients eligible for surgical resection [8]. Several scores to predict recurrence after CRLM resection have been proposed to improve the diagnosis and prognosis of these patients [9–12], with the Fong score being one of the most known tools used for this purpose. However, current risk scoring systems do not accurately reflect the prognosis, and patient survival can be very similar even with very different score values [13]. Thus, identification of a more accurate system to predict patient survival is still of major relevance. In this regard, the use of biomarkers that could, independently or associated with classical prognostic indices, predict the neoplastic evolution of the patient after surgical CRLM resection is a well-defined strategy to enhance the reliability of predicting the prognosis of CRLM [14,15]. Furthermore, biomarkers could improve the selection of the optimal treatment for each patient to enhance their prognosis.

In the current investigation, high-throughput proteomic techniques were used to identify a potential protein panel in human hepatic tumor samples to detect CRLM patients with a poor prognosis. Ultimately, we aimed to validate whether the protein panel profile identified could be useful as a prognostic tool in CRLM patients.

Patients and Methods

Patients and Routine Laboratory Tests

Patients admitted to the Liver Transplantation Unit to undergo CRLM resection from April 2005 to March 2012 were prospectively considered for this study and were followed according to institutional guidelines [16]. We obtained all clinical and pathological data from the Liver Surgery Unit prospective database and clinical charts and selected only the patients who could be included in a specific prognostic group according to survival time. Thus, a total of 85 CRLMs were studied. Exclusion criteria were previous CRLM resection or refusal to participate in the study. Chemotherapy regimens used before hepatic resections were based on 5-fluorouracil alone or associated with irinotecan or oxaliplatin according to local protocols. Patients were classified based on their long-term outcome. Thus, patients were considered as "mild" if their survival time was longer than 5 years or "severe" if it was lower. Venous blood samples from all CRLM patients were obtained after fasting. Extracted serum samples were kept at −80°C, and several serum parameters, including alanine transaminase, aspartate aminotransferase, albumin, bilirubin, or creatinine, among others, were measured with the ADVIA 2400 Instrument (Siemens Healthcare Diagnostics, Tarrytown, NY). CEA serum levels were determined using the ADVIA Centaur XP Immunoassay System (Siemens Healthcare Diagnostics).

The design of the study was two-fold. Firstly, in a training set of 57 CRLM patients, we assessed whether the tissue proteomic profile of the mild patients differed from that of severe patients. Routine liver and renal function tests were also analyzed in these patients. Thereafter, liver tissue samples from 28 CRLM patients were also collected and included in the blinded validation group to confirm the results obtained.

Liver Resection of Metastatic Colorectal Cancer and Specimen Collection

All metastatic tissue samples from patients undergoing curative resection of CRLM were collected and immediately cryopreserved in liquid nitrogen and kept until further analysis.

Hepatic Protein Extraction and Protein Fractionation from Liver Homogenates

Approximately 50 mg of tumor hepatic tissue was ground to fine powder in dry ice and solubilized by pestle homogenization in 500 µl of urea buffer (9.5 M urea, 2% CHAPS, 1% DTT, 50 mM Tris-HCl, pH). Thereafter, tissue homogenates were incubated in a rotating mixer for 1 hour at 4°C, and insoluble material was removed by centrifugation (16,000 ×*g*, 4°C for 15 minutes). Afterwards, samples were aliquoted and kept at −80°C until protein fractionation was performed. In short, samples were fractionated by pH using a 96-well filtration plate (Pall Corp., Port Washington, NY) with 200 µl of a macro-prep high Q anion exchange support (Bio-Rad Laboratories, Hercules, CA) in each well. Flow-through was discarded by vacuum filtration. Subsequently, each well was washed twice with 200 µl of distilled water and equilibrated with 200 µl of rehydration buffer (50 mM Tris-HCl, pH). Wells were incubated for 1 hour at room temperature. Thereafter, each well was equilibrated three times with 200 µl of an equilibration buffer [1 M urea, 0.2% (w/v) CHAPS, 50 mM Tris-HCl, pH]. Prior to fractionation, 20 µl of tissue homogenates was mixed with 30 µl of urea buffer [9 M urea, 2% (w/v) CHAPS, 50 mM Tris-HCl, pH] in a 96-well V-bottom plate for 20 minutes at 4°C in a horizontal orbital microplate shaker. Samples were then diluted with 50 µl of equilibration buffer, transferred to each well, and incubated for 30 minutes at 4°C with shaking. All samples were eluted in a stepwise manner by altering the pH of the wash buffer. Six different fractions were obtained. Flow-through was collected by vacuum filtration into V-bottom microplates, and all were stored at −80°C until proteomic analysis.

Proteomic Processing of Hepatic Tissue Samples

Protein profiling was analyzed by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) using the eight-spot format ProteinChip array (Bio-Rad). In a preliminary study to optimize the experimental conditions, two pooled samples from mild CRLM patients and severe CRLM patients were fractionated, and the six fractions obtained were loaded onto three different types of ProteinChip arrays that had different protein binding affinity; weak cation exchange arrays (CM10), immobilized metal affinity chromatography arrays (IMAC30), and hydrophobic/reverse-phase arrays (H50). The resulting protein profile from each pool was compared, and the fraction number 3 using the CM10 ProteinChip array showed the highest number of peaks detected, the highest total signal intensity, and the major differences between groups when compared with all other fractions and ProteinChip arrays. Thus, fraction number 3 and CM10 ProteinChip array were selected for the subsequent studies. Prior to sample loading, spots were equilibrated twice with 200 µl of a sodium acetate buffer (0.1 M