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Comparative proteomic analysis of human malignant ascitic fluids for the development of gastric cancer biomarkers

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ABSTRACT

Objectives: Malignant ascites is a sign of peritoneal seeding, which is one of the most frequent forms of incurable distant metastasis. Because the development of malignant ascites is associated with an extremely poor prognosis, determining whether it resulted from peritoneal seeding has critical clinical implications in diagnosis, choice of treatment, and active surveillance. At present, the molecular characterizations of malignant ascites are especially limited in case of gastric cancer. We aimed to identify malignant ascites-specific proteins that may contribute to the development of alternative methods for diagnosis and therapeutic monitoring and also increase our understanding of the pathophysiology of peritoneal seeding.

Design & methods: First, comprehensive proteomic strategies were employed to construct an in-depth proteome of ascitic fluids. Label-free quantitative proteomic analysis was subsequently performed to identify candidates that can differentiate between malignant ascitic fluilds of gastric cancer patients from benign ascitic fluids. Finally, two candidate proteins were verified by ELISA in 84 samples with gastric cancer or liver cirrhosis.

Results: Comprehensive proteome profiling resulted in the identification of 5347 ascites proteins. Using label-free quantification, we identified 299 proteins that were differentially expressed in ascitic fluids between liver cirrhosis and stage IV gastric cancer patients. In addition, we identified 645 proteins that were significantly expressed in ascitic fluids between liver cirrhosis and gastric cancer patients with peritoneal seeding. Finally, Gastriscin and Periostin that can distinguish malignant ascites from benign ascites were verified by ELISA.

Conclusions: This study identified and verified protein markers that can distinguish malignant ascites with or without peritoneal seeding from benign ascites. Consequently, our results could be a significant resource for gastric cancer research and biomarker discovery in the diagnosis of malignant ascites.

1. Introduction

Gastric cancer is one of the most lethal malignancies and is diagnosed in nearly 1 million people annually [1]. The predominant treatments for gastric cancer are surgery and endoscopic mucosal resection (EMR) with or without adjuvant systemic chemotherapy. There are no specific drugs that prevent or impede the progression of gastric cancer. Most gastric cancers worldwide are diagnosed in the advanced stages due to vague and nonspecific symptoms, except in the few countries that have a screening program, such as Korea and Japan [2].

Malignant ascites is a sign of peritoneal seeding, which is one of the most frequent forms of incurable distant metastasis, and it is often difficult to diagnose with various tools, such as computed tomography, magnetic resonance imaging, and positron emission tomography, unless it generates a significant amount of seeding nodules or ascites. Occasionally, a small quantity of ascites that results from peritoneal seeding is unable to be distinguished from that under benign conditions, such as portal hypertension, and even from physiological ascites that is related to menstrual cycles [3].

Thus, the recent American Joint Committee on Cancer staging system has classified gastric adenocarcinoma with a positive intraperitoneal cytology as M1 disease, and an intraoperative peritoneal

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cytological examination has become essential for staging advanced gastric cancer [4]. Because the conventional cytological examination is insufficiently sensitive and takes a long time, RT-PCR methods that detect tumor markers and cytokeratins have been examined to increase the sensitivity and speed [5]. These approaches appear to have greater sensitivity, but only a few well-known molecular markers have been studied for detection of free cancer cells [5]. Thus, discovery of new molecular markers that have high sensitivity and specificity is needed to improve the detection rate. Discovery of novel molecular markers, including diagnostic and therapeutic targets, using RT-PCR is time-consuming, expensive, and relatively laborious compared with other omics-based approaches. Therefore, diagnostic and prognostic biomarkers that discovered in body fluids of gastric cancer patients using proteomics and genomics have the potential to improve the diagnosis and treatment of patients with gastric cancer [6,7].

Ascitic fluids is a valuable source of cancer biomarkers, because it contains a variety of secreted and shed proteins from cancerous cells. The principal causes of ascites in western countries are liver cirrhosis (80%) and malignant tumors (10%) [8]. Further, the probability of obtaining ascites due to cancer-related factors is higher in Korea than in the western world [8]. The malignant ascites develops in the advanced stages of gastric cancer, when metastases form in the peritoneal cavity due to neovascularization, angiogenesis, increased fluid filtration, and lymphatic obstruction [9]. Because the development of malignant ascites is associated with a very poor prognosis, determining whether it resulted from peritoneal seeding is critical with regard to the diagnosis, selection of the appropriate treatment, and therapeutic monitoring [10]. An accurate diagnosis obviates the need for extensive surgery, which is not beneficial for patients and can even result in harmful postoperative complications. It is also important to implement a systemic chemotherapy strategy as a palliative measure instead of as an adjuvant treatment. Thus, exploiting ascites by systemic proteomics to identify cancer-specific signatures can guide the development of alternative methods for diagnosis and therapeutic monitoring and increase our understanding of the pathophysiology of peritoneal seeding [10,11].

Conversely, most proteomic studies on ascitic fluids [12] have focused on ovarian cancer and provided insufficient depth for biomarker discovery. Further, no proteomic analysis of ascitic fluids from gastric cancer has been reported. To this end, we profiled the human ascites proteome to obtain a pool of biomarker candidates. Consequently, label-free quantitation was performed to compare the proteomes between benign disease and gastric cancer patients with peritoneal seeding.

2. Materials and methods

2.1. Ascitic fluids sample collection

We collected 50 ml of ascites per patient, who was diagnosed with benign disease (n = 27) or stage IV gastric cancer (n = 85). All patients provided informed consent before being enrolled per the protocol that was approved by the institutional review board of Korea National Cancer Institute (IRB No: NCCNCS-12-581). A total of eight samples contaminated with blood were excluded. To further verify our findings in label-free quantification, we performed the enzyme-linked immunosorbent assay (ELISA) using 27 benign samples and 57 malignant ascitic fluids samples (Supplementary Table S1 and S2).

2.2. Ascitic fluids samples preparation

To remove high-abundance proteins, pooled ascitic fluids samples were depleted using a MARS-6 column (Agilent, CA, USA). The protein concentrations in un-depleted and depleted ascitic fluids were measured by the BCA assay. For in-depth profiling, proteins were digested by in-gel digestion, UREA in-solution digestion, and filter-aided sample preparation (FASP) as previously described [13]. Prior to peptide fractionation or LC-MS/MS analysis, all digested peptide mixtures were acidified with 1% TFA and desalted using an OASIS C18 column or homemade StageTips [13]. Desalted samples were lyophilized in a speed-vacuum centrifuge and stored at −80 °C before peptide fractionation or LC-MS/MS analysis. Peptides were fractionated by strong cation-exchange (SCX) chromatography or StageTip-based high-pH reversed-phase fractionation [13]. Detailed procedures for the sample preparation and peptide fractionation are described in Supplementary Methods.

2.3. LC-MS/MS analysis & MS data processing

The non-fractionated (1 μg) or fractionated peptides (1 μg per fraction) were analyzed by online nanoflow liquid chromatography-tandem mass spectrometry (LC–MS/MS), consisting of an Easy-nLC 1000and a Q-Exactive quadrupole Orbitrap mass spectrometer, as described with some modifications [13]. Each sample was analyzed in triplicate or quadruplicate (single-shot) for technical replications. For the label-free quantitation, fractionated samples were analyzed in a single technical replicate.

For in-depth profiling using 4 approaches, database searches were performed in Proteome Discoverer (Thermo Fischer Scientific, ver 1.4) using Sequest-HT and MSAmanda. The MS/MS data were queried against the UniprotKB human protein database (2016 august released) [14]. Peptide identifications were filtered using Percolator, based on q-values at a 1% FDR [15].

For the label-free quantitation, database searches were performed as described above. For each precursor ion, the peak area was calculated from the ion chromatogram that was extracted using the precursor ion area detector node. The protein abundance in each sample was calculated as the sum of all identified peptide peak areas for a given protein. All downstream analyses for the label-free quantitation were performed using Perseus software.

The gene ontology (GO) term in the protein datasets were analyzed using the DAVID bioinformatics resource tool (http://david.abcc.ncifcrif.gov/) and the UniprotKB database (http://www.uniprot.org/) [14]. Detailed information on the LC-MS/MS analysis and MS data processing in provided in Supplementary Methods. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium [16] via the PRIDE partner repository with the dataset identifier PXD002213 (single-shot, gel-based, and SCX fractionation), PXD003351 (high-pH fractionation), and PXD004501 (label-free quantification).

2.4. ELISA

The verification assay was performed by ELISA according to the manufacturer's instruction. After determining the optimal ascites dilution factor for each protein, the concentration of Gastriscin (PGC) and Periostin (POSTN) were measured and quantified in ascitic fluids (benign disease: 27 and gastric cancer: 57). Statistical analysis (independent t-test & AUC analysis) was performed using MedCalc program (MedCalc Software bvba, Ostend, Belgium).

3. Results

3.1. In-depth profiling of human ascitic fluids

To obtain a comprehensive proteome in human ascites, we implemented 4 proteomic strategies using various analytical fractionation methods, based on high-resolution mass spectrometry. First, we pooled samples (B- and C-Pool 1) from individuals with benign diseases and gastric cancer who were randomly selected from the cohort (N = 8 per each replicate). Combined with immune-affinity depletion and peptide fractionation methods, including single-shot, SDS-PAGE, SCX, and high-