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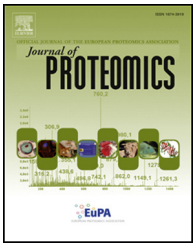

Longitudinal study of circulating protein biomarkers in inflammatory bowel disease

Emilie Viennois[a,b,c,*], *Mark T. Baker*[a,b], *Bo Xiao*[a,b], *Lixin Wang*[a,b,c], *Hamed Laroui*[b], *Didier Merlin*[a,b,c]

[a]*Institute for Biomedical Sciences, Georgia State University, Atlanta, GA 30303, USA*
[b]*Chemistry Department, Center for Diagnostics and Therapeutics, Georgia State University, Atlanta, GA 30303, USA*
[c]*Veterans Affairs Medical Center, Decatur, GA, USA*

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ABSTRACT

Inflammatory bowel diseases (IBDs) are chronic and progressive inflammatory disorders of the gastrointestinal tract. In IBD, protein serological biomarkers could be relevant tools for assessing disease activity, performing early-stage diagnosis and managing the treatment. Using the interleukin-10 knockout (IL-10$^{-/-}$) mouse, a model that develops a time-dependent IBD-like disorder that predominates in the colon; we performed longitudinal studies of circulating protein biomarkers in IBD. Circulating protein profiles in serum samples collected from 30-, 93-, to 135-day-old IL-10$^{-/-}$ mice were investigated using two-dimensional differential gel electrophoresis and MALDI-TOF/TOF tandem mass spectrometry. A total of 15 different proteins were identified and confirmed by ELISA and Western blot to be differentially accumulated in serum samples from mid- to late-stage IL-10$^{-/-}$ mice compared to early non-inflamed IL-10$^{-/-}$ mice. The use of another model of colitis and an extra-intestinal inflammation model validated this biomarker panel and demonstrated that comprised some global inflammatory markers, some intestinal inflammation-specific markers and some chronic intestinal inflammation markers. Statistical analyses using misclassification error rate charts validated the use of these identified proteins as powerful biomarkers of colitis. Unlike standard biomarker screening studies, our analyses identified a panel of proteins that allowed the definition of protein signatures that reflect colitis status.

Biological significance
Crohn's disease (CD) and ulcerative colitis (UC) are the most common inflammatory bowel diseases (IBDs) occurring in humans. The major current diagnosis tool is colonoscopy, which is invasive and could lead to false diagnosis. The emergence of serological biomarkers enables the use of new diagnosis tools such as protein signatures for IBD diagnosis/management. Using 2D-DIGE coupled to mass spectrometry, our longitudinal study in a mouse model of colitis identified a signature of protein biomarkers for specific stages of disease.

* *Corresponding author at:* Institute for Biomedical Sciences, Georgia State University, Atlanta, GA 30303, USA. Tel.: +1 404 413 3598; fax: +1 404 413 3580.
E-mail address: eviennois@gsu.edu (E. Viennois).

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1. Introduction

Inflammatory bowel diseases (IBDs) are chronic inflammatory conditions of the gastrointestinal (GI) tract that affect about 1.4 million people in the United States and approximately 2.2 million people in Europe [1,2]. Two major clinical forms of IBD have been extensively studied: Crohn's disease (CD) and ulcerative colitis (UC). In CD, inflammation can occur anywhere in the GI tract, even if it primarily affects the ileum, whereas in UC, the colonic mucosa is principally involved [3]. Both diseases are thought to feature alterations in the immune response to GI microbiota in individuals genetically predisposed to IBD alterations characterized by intestinal epithelial barrier disruption and an influx of immune cells [4].

IBD is a substantial public health problem whose efficient assessment remains a difficult challenge. The clinical diagnosis of IBD is achieved through colonoscopy, possibly complemented by parallel analyses of some blood markers. Serological biomarkers are already in use to assess IBD activity, provide early stage diagnosis, evaluate prognosis, and monitor remission stage. Notable among currently available biomarkers are serum C-reactive protein (CRP) and calprotectin [5,6]. However, CRP and calprotectin levels are increased under any inflammatory condition [7,8], and thus are not specific for inflammation of intestinal origin, significantly limiting their usefulness in IBD. Effective diagnosis and surveillance of complex multi-factorial disorders, like IBD, could be improved through screening of easily accessible biomarkers, such as circulating serum protein, using a more defined expression pattern capable of discriminating the degree of inflammation. Such an approach could improve prediction of IBD progression, which remains a substantial challenge. Furthermore, the development of reliable non-invasive biomarkers would be highly valuable for earlier detection, which is extremely important for treatment effectiveness, and thus could prove beneficial for the treatment of IBD patients and monitoring their response to treatment.

Multiple animal models of IBD have been developed [9]. Although these models do not adequately recapitulate the full complexity of the human disease, they are nonetheless valuable and indispensable tools, providing a wide range of options for investigating the involvement of various factors in the pathogenesis of IBD. Importantly, they can be used to evaluate different therapeutic options that cannot be investigated in humans. The use of a mouse model for IBD biomarker discovery also allows easy access to large numbers of samples from a uniform genetic background, a controlled environment and uniform sample collection.

One of the most commonly used mouse models of intestinal inflammation is the interleukin-10 knockout (IL-10$^{-/-}$) mouse model. IL-10 is a potent suppressor of macrophage activation in vitro. It inhibits the production of inflammatory cytokines such as IL-1, IL-6, and tumor necrosis factor α (TNF-α) by macrophages stimulated with lipopolysaccharide (LPS) or interferon-γ (IFNγ) [10]. Mice with a targeted deletion of the IL-10 gene, first generated and phenotyped in 1993, spontaneously develop chronic enterocolitis with massive infiltration of lymphocytes, activated macrophages, and neutrophils in a Th1 cell-mediated manner [11,12]. In IL-10$^{-/-}$ mice, lymphocyte development and antibody responses are normal, but most animals exhibit growth retardation and anemia, and suffer from chronic enterocolitis. Alterations in the intestine include extensive mucosal hyperplasia, inflammatory reactions, and aberrant expression of major histocompatibility complex class II molecules on epithelia [12]. The predictability of the timing of colitis in IL-10$^{-/-}$ mice allows longitudinal assessment of blood samples at various stages of colitis progression.

Because serum is an easily accessible source material, it plays a significant role in proteomic approaches designed to identify biomarkers for the early detection of numerous diseases. Proteomics is a powerful and an effective approach for rapidly evaluating protein profiles in serum. One frequently used proteomic tool is two-dimensional differential gel electrophoresis (2D-DIGE), an advanced, sensitive gel-based separation and quantification approach in which protein samples are pre-labeled with different fluorescent dyes, mixed, and run simultaneously on the same gel. One of the greatest challenges for proteome analysis is the reproducible fractionation of complex protein mixtures while still retaining the ability to qualitatively and quantitatively address the relationships between two or more samples. To date, two-dimensional gel electrophoresis is the only method that allows a suitable separation of complex protein mixtures and provides the means for the accurate and reproducible visualization of their expression profile. 2D-DIGE and the use of CyDye chemistry further allow the separation and quantitative analysis of two or more different protein samples within the same gel, thus minimizing any gel to gel variations. Two-dimensional gel electrophoresis coupled with mass spectrometry (MS) is a typical proteomic approach for the identification of new biomarker candidates [13,14]. However, to date, no studies have used 2D-DIGE for the analysis of serum in the context of IBD biomarker discovery.

Here, using 2D-DIGE and MS, we identified a total of 15 proteins that were differentially accumulated in serum samples from mid- to late-stage colitis in IL-10$^{-/-}$ mice compared to early non-inflamed IL-10$^{-/-}$ mice. Unlike traditional biomarker screening studies, our analysis identified a panel of proteins that allowed the definition of different protein signatures that reflect colitis status.

2. Materials and methods

2.1. Mice

Three week-old female C57BL/6 and IL-10$^{-/-}$ mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were group housed under a controlled temperature (25 °C) and photoperiod (12:12-h light–dark cycle) and allowed unrestricted access to standard mouse chow diet and tap water. All studies were performed in accordance with the Institutional Animal Care and Use Committee at Georgia State University (Atlanta, GA). All procedures were approved and are registered in the protocol IACUC ID: A11025 (approval date from 8/30/2011 to 8/30/2014).

2.2. Sample collection and preparation

Blood samples were collected in serum separator tubes (BD Microtainer) by retro-orbital puncture from IL-10$^{-/-}$ mice at