INVITED REVIEW

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Update on current applications of proteomic in the study of inflammatory bowel disease

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Abstract

Ulcerative colitis and Crohn's disease are relapsing and remitting chronic disorders. So far, endoscopy is the gold standard for their diagnosis, but less invasive diagnostic biomarkers are needed. Many authors have developed techniques to individuate biomarkers such as genetic testing factor or proteins in biological samples such as serum, plasma, and cellular subpopulations. A protein fingerprint pattern, patient-unique, specific for the diagnosis of inflammatory bowel disease (IBD) and potentially able to predict the future patterns of disease and to help in diagnosis, treatment, and prognosis is of increasing interest among researchers. Nowadays, a proteomic approach may be used in the identification of major alterations of proteins in IBD, but there is still a lack in the identification of a panel of biomarkers among a significant number of patients in large clinical trials. In this review, we analyze and report the current knowledge in proteomic application and strategies in the study of IBD.

Keywords Crohn's disease, ulcerative colitis, proteomic, biomarkers, metabolomic, nutrigenomic

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Introduction

Inflammatory bowel disease (IBD), i.e., ulcerative colitis (UC) and Crohn's disease (CD), are chronic inflammatory diseases, the pathogenesis of which is the result of altered immunological responses in a genetically susceptible host. Recently, the gut-associated microbial ecosystem has been explored as an important active player in IBD pathogenesis [1].

Nowadays, diagnosis of IBD is performed by endoscopic, histological, and radiographic tests, but even when performed by expert clinicians, they can result in diagnostic uncertainty. Moreover, early diagnosis is relevant in the therapeutic decision and thus in modifying the natural history of a disease.

None of the markers available have enough high sensitivity and specificity to allow an early and differential diagnosis between CD, UC and other colitis [2-38].

In the last decade, new technologies, such as genomics and proteomics, have combined in the study of IBD. The knowledge of the genes involved in the etiopathogenesis of IBD and their products is of great interest for clinical use. Other -omic technologies, like metabolomics and nutrigenomics are emerging as new potential approaches in the discovery of new targets for the development of new drug therapies.

Many authors using new advanced methodologies such as proteomics but also metabolomics, nutrigenomics and subproteomics, have explored serological markers. The latter technique, besides investigating the proteome of different biological fluids, introduces the concept of functional proteomics and therefore might be of relevance because it focuses on the different cell compartment contents. Clinical subproteomics is achieving greater importance in the identification of IBD-related protein profiles or biomarkers. Moreover, new technologies for understanding the crosstalk between the microbiota and the host are emerging.

The aim of this review is to critically report the new bioanalytical tools in terms of suitability and real applicability to IBD biomarker discovery, reviewing the "state of the art" proteomic discovery biomarkers in IBD.

Proteomics

Analytical approaches

Mass spectrometry (MS) measures the mass to charge ratio (m/z) of ionized analytes, such as proteins or peptides. MS

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can be equipped with different ionization sources and mass analyzers. The most common are electro-spray ionization and matrix-assisted laser desorption ionization (MALDI) MS, while hybrid analyzer combining quadrupole and time-of-flight (TOF) are predominant [39]. Separation techniques play a pivotal role in the simplification of the sample. Proteins are separated on the basis of their molecular weight and isoelectric point using gel electrophoresis. It is the first protein separation technique that could be combined with MS. It allows cutting a single spot or band of the gel that is digested by proteases. This approach permits to perform qualitative and quantitative analysis at the protein level and to investigate post-translational modifications.

Liquid chromatography is another technique able to separate peptides prior to MS analysis. This procedure is faster and more reproducible than 2D-electrophoresis, it provides the identification of a larger amount of proteins. Quantitative analysis can be performed by a label-free approach or by labeling with isotopic peptides. Label-free quantitation is performed by comparison of the signals of the peptides eluted during the chromatographic separation. The performance of this approach can be affected by shifts in the retention time of the peptides between different runs. Isotopic labels can be distinguished among label strategy in enzymatic, metabolic, and chemical reactions. Enzymatic labeling can be performed during or after proteolytic digestion with proteases, while metabolic labeling can be applied to cell cultures by addition of isotope-enriched amino acids to the medium culture. Chemical label is the only label that can be applied to every type of biological sample [40-43]. These analytical approaches have been applied to a large variety of samples such as serum and plasma, freshly isolated cells, tissue and cell line in order to investigate the etiopathogenesis of IBD (Table 1).

Understanding the complexity of the data obtained by the analytical approaches described above is the goal of bioinformatics. Bioinformatics provides new algorithms to manage the large and heterogeneous amount of data such as new algorithms for image analysis of two-dimensional gels and for peptide mass fingerprinting and peptide fragmentation fingerprinting. Thus, bioinformatics emerges as an important approach to proteomic data sets, in order to understand the diversity between the normal and abnormal cell proteome of various biological systems.

Serum proteomics

Proteomics may contribute to biomarker discovery because it identifies a panel of proteins suitable as biomarkers in each biological sample [44-46].

Several groups have used serum for proteomic studies because of its simplicity. Meuwis first described several markers in IBD serum using surface-enhanced laser desorption/ionization /TOF MS such as platelet aggregation factor 4 (PAF4), myeloid-related protein 8, fibrinogen-α, and haptoglobin a2 (Hpa2). Moreover, they identified a correlation between PAF4 and the response to anti-tumor necrosis factor alpha (anti-TNF-α) therapy. They demonstrated that anti-TNF-α non-responding CD patients have higher concentrations of PAF4 factor than healthy subjects [47,48]. Using different selective solid-phase bulk extraction, MALDI TOF MS and chemometric data analysis, we have found 20 proteins able to discriminate between healthy controls, CD and UC serum samples [49].

Cell proteomics

In normal condition, gut homeostasis derives from the cooperation of different populations of cells located throughout the mucosa and submucosa. From these interactions protein networks derive and create new interactions and cellular modifications.

Different proteomics approaches have been applied to *in vitro* models of IBD (colonic epithelial cell lines) as well as to cells derived from human samples and to different mouse models of colitis [50-52]. Hardwidge *et al* performed a large-scale proteomic study using liquid chromatography and MS to evaluate the response of colonic cell line to the enteropathogenic *Escherichia coli*. They described more than 2,000 proteins differently expressed in the presence or absence of the pathogen [52]. Several targets of inflammatory cytokines downstream pathway (tryptophanyl-tRNA synthetase, indoleamine-2,3-dioxygenase, heterogeneous nuclear ribonucleoprotein JKTBP, interferon-induced 35 kDa protein, proteasome subunit LMP2, and arginosuccinate synthetase) have been described in freshly isolated intestinal epithelial cells (IECs) samples from IBD patients. Overexpression of indoleamine-2,3-dioxygenase, an enzyme involved in tryptophan metabolism, was found in CD as well as in UC IECs [50,51].

Several proteins, like Annexin 2A, involved in cell death, signal transduction and energy metabolism, and induced as a response to stress, were found upregulated in inflamed area from UC IECs samples [53] and the their increase correlated with inflammation and repair mechanisms. For example, protocadherins play a role in maintaining orderly growth during the re-epithelialization process, since they were found linked to the retention of the monolayer morphology of proliferating cells [54]. Hsieh *et al* using bidimensional electrophoresis (2DE) and MS, identified differences in the expression of proteins among active and inactive UC such as mitochondrial proteins, proteins involved in energy generation, or stress-response proteins [55]. Because of the need for information concerning the pathogenesis of IBD, the variations in the protein expression of lymphocytes and mucosa were studied in rats with induced colitis and CD and UC patients. Recently, the protein profile of lymphocytes from an antigen-specific model of colitis has been performed using proteomic approaches. Liu *et al* identified 26 altered proteins in lymphocytes isolated from rat with 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis. Among these proteins some are involved in inflammation, such as myeloid-related protein 14, a potential mediator of p38 mitogen-activated protein kinases -dependent functional responses, apoptosis,

metabolism, such as ATP-citrate synthase, regulation of cell cycle, cell proliferation, signal transduction, such as nucleoside diphosphate kinases, and ubiquitin conjugating enzyme E2N [56].

Direct tissue analysis

Histology-directed protein profiling allows acquiring spectra from areas of mucosa or submucosa within a single tissue section without preparation of a sample.

Berndt's group performed a proteomic analysis of inflamed and uninflamed areas of the gut from IBD patients using a novel automated multidimensional, fluorescence-based microscopy robot technology demonstrating that IBD has a greater number of CDAþ CD7-memory T cells [57].

Recently, other groups have used the ability of MALDI to perform histology-directed cellular protein analysis of tissues. M'Koma analyzed mucosal and submucosal layers of CD and UC colon resection samples after histologic assessment. MALDI-MS appeared to be capable to distinguish CD and UC while profiling the colonic submucosa [58].

Subproteomics

The biological fluids and cells are very complex samples composed of thousands of peptides, proteins, and products of metabolism. Subproteomic is crucial in determining a protein profile of a single subset of cells or a single compartment by applying new strategies for the fractionation, separation, and enrichment of proteins derived from a specific compartment or cell subtype.

Examples of subproteomes that are being studied include large macromolecular complexes and cellular machines, specific classes of proteins and organelles. Although the same analytical technologies may usually be employed for global and targeted proteomic studies, the latter studies require specific initial strategies to isolate the appropriate subproteome components.

Subcellular proteomics

Huber *et al* introduced the concept of subcellular proteomics. Subcellular fractionation is a flexible approach that reduces sample complexity and is efficiently combined both with high-resolution 2D gel/MS analysis and with gel-free independent techniques [59]. Subcellular fractionation allows access to intracellular organelles and multiprotein complexes and tracking proteins that shuttle between different compartments (e.g., between the cytoplasm and nucleus) [60]. Subproteomics represents a relevant approach to the study of IBD where there are defects in different compartments. Rectal biopsy specimens from control subjects and from patients with CD, non-rectal CD, and acute UC were subjected to sucrose density gradient centrifugation. The activity of enzymes such as 5'nucleotidase (plasma membrane), malate dehydrogenase (mitochondria), catalase (peroxisomes), lactate dehydrogenase (cytosol), N-acetyl-beta-glucosaminidase (lysosomes), neutral-alpha- glucosidase (endoplasmic reticulum), were tested [61].

We evaluated the proteome of the subcellular fractions (nuclei, membranes, and cytosols) of IECs isolated from healthy subjects and CD, using a label-free liquid chromatography-MS approach. We found in CD an increase in proteins such as heat shock 70 kDa protein 5, tryptase alpha-1 precursor, and proteins whose upregulation can be explained by the increased activity of IECs in the inflammatory state. A lower abundance in CD of proteins such as Annexin A1, a mediator of the antiinflammatory actions of glucocorticoids, and malate dehydrogenase was found too [62].

Serum subproteomics: microparticles

Recent studies have focused on the relationship between inflammation and blood coagulation in the pathogenesis of IBD, where there is an increase in the number of circulating platelets.

In CD, platelet activation is demonstrated by positivity for P-selectin (CD62P), b-thromboglobulin, and platelet products such as platelet factor 4 (PF4) and CD40 ligand (CD40L). Activated platelets produce platelet-derived microparticles (PDMPs) in response to different stimuli. PDMPs range in size from 0.02 to 0.5 lm and carry several antigenic characteristics of intact platelets such as glycoprotein (GP) IIb/IIIa, GPIb/IX, and P-selectin (CD62P). The generation of microparticles can be the consequence of proinflammatory cytokines production [63-69].

The PDMPs are membrane-derived microvesicles (MVs) released from the cell surface and implicated in cell-to-cell communication. MVs may represent vehicles that can facilitate interaction between target cells by surface expressed ligands; they can transfer surface receptor, deliver proteins, mRNA, intact organelles, and modulate the cell-to-cell network. Huber's group investigated the role of microvescicles secreted from colon cancer cells in inducing T cell apoptosis and escaping the immune system. They demonstrated that tumor microparticles are able to induce the apoptosis of activated CD8þ T cells through the expression of both a granular pattern of tumor necrosis factor-related apoptosis-inducing ligand and the Fas ligand [70].

Our group has speculated that PDMPs from CD patients contain specific esopeptidase which involves the fibrinopeptide as substrate [71].

From metabolomics to nutrigenomics

Metabolomics is the "systematic study of the unique chemical fingerprints that specific cellular processes leave behind"-specifically, the study of their small-molecule metabolite profiles. The metabolome represents the collection of all metabolites in a biological organism, which are the

end products of its gene expression. Metabolic profiling can give an instantaneous snapshot of the physiology and pathophysiology of that cell.

The urinary metabolomic profiles in interleukin 10 gene-deficient mice using high performance liquid chromatography - MS revealed that changes in trimethylamine and fucose correlates with disease activity [72]. Marchesi characterized fecal extracts using a noninvasive metabolomics approach, which combines high-resolution 1H NMR spectroscopy and multivariate pattern recognition techniques. The fecal extracts from IBD patients were characterized by reduced levels of butyrate, acetate, methylamine, and trimethylamine in comparison with a control population, suggesting changes in the gut microbial community. Elevated quantities of amino acids were present in the feces from both disease groups. The roles that amino acids play in immunity and inflammation are well defined, and the relationship between IBD and certain amino acids has recently been studied [73]. Ooi *et al* studied the levels of amino acids and trichloroacetic acid (TCA) cycle-related molecules in the colonic tissues and sera of patients with UC by gas chromatography MS. They observed lower levels of 16 amino acids and 5 TCA cycle-related molecules in UC patients than in CD patients and healthy subjects [74].

The role of bacteria in the pathogenesis of several gastrointestinal disorders is well established. Kumari first introduced the concept of quorum sensing in IBD. Altered bacterial balance in the gut may lead to inflammation. Bacteria create communications among themselves (quorum sensing) to maintain their behavior and colonization. Quorum sensing enables bacteria to communicate with each other and control expression of certain specialized genes by producing and responding to extracellular signals in proportion to cell density.

Kumari proposed a method based on genetically engineered whole-cell sensing systems to detect quorum-sensing molecules in biological samples and to use these molecules as biomarkers [75].

Nutrigenomics is defined as "a transdisciplinary approach to understand the subtle but contentious impact of nutrition as prime environmental trigger in shaping the dynamic range between health and disease". Proteomic analysis of primary intestinal epithelial cells, as mentioned above, has confirmed a network of differentially regulated proteins clustered around mithocondrial stress mechanisms. Different authors have shown a connection between mithocondrion and endoplasmic reticulum in triggering stress-associated unfolded protein response that could implement inflammation. Nutrigenomic together with proteomic and genomic could represent new progress in understanding the network between diet-gene and/or diet-microbe interaction and to discover new links between metabolome and IBD pathogenesis [76].

Conclusions

In this review we have summarized the current "state of the art" in emerging proteomic technologies and their applications in the study of IBD. Despite the fact that new advanced tools are available, few groups have attempted to apply proteomic study for the discovery of new biomarkers in IBD.

The complex etiopathogenesis of CD and UC which are the result of modifications occurring at different levels may be revealed by the study of protein modification using proteomic and the related technologies. Moreover, because several cell subpopulations, compartments are affected, subproteomics may allow target proteomic studies.

Furthermore, the emerging role of microbiota in maintaining the intestinal homeostasis point out the need to integrate proteomics together with metabolomics and nutrigenomics. Thus, understanding the diet-host-microbiota interactions may help to identify novel targets in the etiopathogenesis of IBD.

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