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Delineation of Inflammatory Bowel Disease by Molecular Biometrics: Verification *versus* Validation

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Abstract

Dysregulated immune responses to the gut microbiota are believed to be the driving force in the development of inflammatory bowel diseases. Even with combined modern and newer diagnostic technologies, it is difficult to accurately differentiate colonic inflammatory bowel disease, namely ulcerative colitis and Crohn's colitis. Regardless of current advanced clinical and pathological criteria available to identify factors possible to help delineate the two entities, a subgroup of Crohn's colitis cases are still diagnosed as ulcerative colitis. Current classification criteria as well as diagnostic modalities such as serologic, genetic and inflammatory markers fail to differentiate ulcerative colitis from Crohn's colitis among patients with indeterminate colitis. The definitive diseases share demographic and clinical features, yet differ in tissue inflammation and damage suggesting distinct trigger and mechanisms. Since treatments differ, a biological dysregulation underlying the two forms of inflammatory bowel disease need to be elucidated in order to develop appropriate treatment strategies that achieve long-term remission and minimize risk of relapse. A molecular diagnostic tool would greatly benefit indeterminate colitis patients. Herewith, we summarize advances and explain the attempts to resolve indeterminate colitis into Crohn's colitis and ulcerative colitis; and ascertain whether Crohn's colitis patients mistakenly operated for definitive ulcerative colitis can molecularly be identifiable prior to surgery.

Keywords: Inflammatory bowel disease; Ulcerative colitis; Crohn's colitis; Indeterminate colitis; de novo Crohn's ileitis; Molecular Diagnostics; Advances and challenges

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Introduction

Colonic inflammatory bowel disease (IBD), "the colitides", include ulcerative colitis (UC) and Crohn's colitis (CC) [1,2]. When state-of-the-art criteria for either are inconclusive, the disease is termed "indeterminate colitis (IC)" [1,2]. IC originally referred to those 15% of cases of IBD in which there was difficulty distinguishing between UC and CC in the endoscopy or colectomy specimen [1-3]. Patients with IC on average are younger than those with either colitides [4,5]. UC and CC share several demographic and clinical features yet present significant differences in tissue inflammation and damage, suggesting a different etiopathogenesis [6]. Theoretically, it is believed that IBD is triggered by inappropriate activation of the gut mucosal immune system against indigenous bacteria in the intestinal lumin [6]. Delineating UC and CC among patient cohort with IC has remained a major challenge in endoscopic medicine [7] and colorectal surgery [8-10]. Disease unpredictability, treatment side-effects, potential surgery, interim morbidity and acute incapacitation are individual concerns and system burdens [11]. Because pharmacological treatments and standard surgical management for the two disease entities are basically different, developing a phenotype-specific molecular biometric tool would allow an accurate, simple, and fast screening diagnostic and/or prognostic test to help clinicians to manage IBD. This tool will be invaluable for IC patients in the clinical setting for precise care [12-14].

Indeterminate Colitis

The need for IC molecular classification into UC and CC phenotype is urgent for patients suffering from IBD [15,16]. Patients diagnosed with IC are relatively young [11] with onset of

Figure 1: Show averaged mass spectrum proteomic pattern spectra from CC and UC. Differential distribution of three selected proteomic pattern peaks (m/z) obtained from colonic mucosal and/or submucosal tissue sections that were part of the Support Vector Machine (SVM) model. Prior to submitting samples for identification, we needed to obtain the exact mass for the proteins of interest. Samples are sectioned at 12uM on the cryostat at -20C. We look for e.g. the following m/z: 3409/ 5655/ 5696/ 6693. The samples are washed in graded ethanol: 70/ 90/ 95 for 30 seconds each. Then samples are spotted using P1 with 20mg/ml SA in 50:50:0.1 ACN:H2O:TFA. Finally, the samples are then submitted for FT analysis. **a**, The pink spectrum corresponds to sample D-4631 (Crohn's colitis), one of the samples used in the Support Vector Machine (SVM) model. **b**, Pink= Spectrum from D-4631 (CC). All other spectra are from WD-12912 (CC).

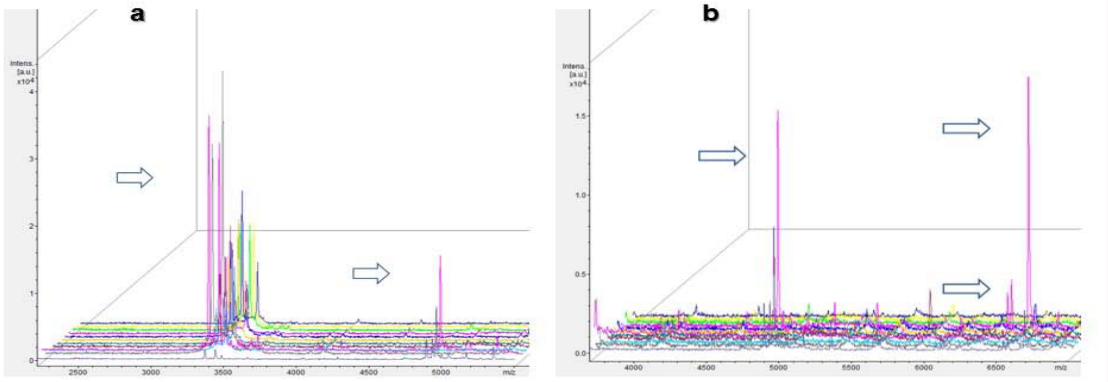

Figure 1:

symptoms before or shortly after 18 years of age [2,17] and have an equal gender distribution [18]. This differs from UC, where there is a male predominance with a mean age of onset at 36-39 years [19]. These figures have remained persistent despite the introduction of combined and modern diagnostic tools [15]. Even after long-term follow-up, a substantial number of patients with IC still retain an unascertained diagnosis [15]. The continued presence of an IC diagnosis over time supports part of our hypothesis that IBD may represent a spectrum of diseases rather than just two entities, CC and UC [20]. Evidently, the IBD incidence and prevalence is increasing worldwide and so are the IC cohorts [21]. In order to resolve diagnostic challenges, an exclusion molecular biometric tool for differential diagnosis is needed now more than ever [20]. However, serological test challenges are still real [14].

Diagnostic Gold Standard

There is no diagnostic gold standard tool for IBD [4,20]. Clinicians use an inexact combined classification system which includes clinical, endoscopic, radiological, and histopathological modalities in attempting to diagnose CC and UC [4,20]. Even with a combination of these approaches, IBD patients are mistakenly diagnosed [15], resulting in inappropriate pharmacologic indications and unnecessary surgical interventions [22]. Restorative proctocolectomy (RPC) with ileal pouch-anal anastomosis (IPAA) has in the past four decades become the most commonly used procedure for treatment of patients with UC [23]. Since its original description, the procedure has been modified in order to obtain optimal functional results with low morbidity and mortality, and yet provide a cure for the disease [24]. The most difficult and painstaking postoperative experience is when patients undergoing a sphincter preserving operation, RPC for definitive UC change in their original diagnosis to *de novo* Crohn's ileitis of the ileal pouch [20]. Disease characteristics of *de novo* ileal pouch Crohn's ileitis heavily influence pouch retention [25]. The interval from pouch construction, fistulizing disease, and disease location are commonly experienced and can be used as prognostic indicators when ileal pouch Crohn's ileitis is diagnosed [25]. Identification of pathologic features associated with "UC-like Crohn's disease (CD) has recently been described [4]. When more severe disease activity is located in the proximal region, distal small bowel involvement, active appendicitis, and prominent lamina propria neutrophils may be morphological factors associated with UC-like CD [4]. A solution to these difficult challenges in misdiagnosis and/ or delayed diagnosis may be achievable through molecular biometric profiling [26-29]. These are challenging but promising studies which are currently underway [15].

Currently, not much is known about the molecular differences distinguishing the colitides [7,8]. Trends in the IBD field have focused on genetic susceptibility, role of indigenous flora, inflammatory processes, and interactions between normal indigenous flora and the immune response [30]. Even though current research is promising [8,15], there have been no definitive solution to help clinicians differentiate between the two disease entities (UC vs. CC) when current diagnostics prove inadequate and result in a diagnosis of IC [4,5]. Rising incidence and prevalence of IBD (Figure 1) across the world [21] is accompanied by an increase in cases of IC [2,3]. It is becoming even more important to find molecular signatures of the colitides to distinguish between CC and UC among patients with IC [7,8].

Supportive Diagnostic Criteria in IBD

Diagnostic criteria of UC, CC and IC diseases can be different between institutions. Our pathologist teams at Meharry Medical College and Vanderbilt University Schools of Medicine uses the following protocol criteria for the final surgical pathology reporting.

For ulcerative colitis

Depending on the phase of disease and the degree of inflammatory activity, UC-related colitis is categorized as chronic inactive, chronic active, or active (without features of chronicity) for the purpose of sign-out. Chronic colitis (regardless of "activity") is defined by the presence of histologic features of chronicity, such as crypt architectural distortion, crypt atrophy, diffuse mixed lamina propria inflammation, basal plasmacytosis, basally located lymphoid aggregates, and Paneth

cell metaplasia (in the left colon). Other changes of chronicity include lamina propria fibrosis, pyloric gland metaplasia, and Paneth cell hyperplasia in the right colon. Common changes of "activity" include neutrophilic or eosinophilic cryptitis, crypt abscesses, regenerative or degenerative epithelial changes, hemorrhage, necrosis, erosions, and ulceration.

For Crohn's disease

The mucosa may show a wide spectrum of changes ranging from completely normal to diffuse and severe chronic active inflammation with ulceration. In biopsies, mucosal disease shows chronic inactive, chronic active or active changes. Histologic features of chronicity include crypt architectural distortion, crypt atrophy, diffuse mixed lamina propria inflammation, basal plasmacytosis, basally located lymphoid aggregates, pyloric gland metaplasia, and Paneth cell metaplasia (in the left colon). Other changes of chronicity include lamina propria fibrosis and Paneth cell hyperplasia in the right colon. "Activity" is characterized by the presence of neutrophilic or eosinophilic cryptitis, crypt abscesses, regenerative or degenerative epithelial changes, necrosis, erosions, and ulceration. The degree of activity is graded as mild if less than 50% of the mucosa shows evidence of activity, moderate if more than 50% of the mucosa shows these features, and severe if surface erosion or ulceration is present.

For intermediate colitis

This entity is used in cases where both inconclusive features of ulcerative colitis and Crohn's disease are present in the some specimen. The cases exhibit extensive disease and the classification is used temporarily until a definitive classification can be established or in cases where following the application of clinical and morphologic criteria a definitive diagnosis is not possible.

Advances

Recently, this laboratory has quantitated the global expression profiles of RNA levels using oligonucleotide microarray/genome-wide transcriptome analysis [31,32] to investigate transcriptional signatures present in colonic tissues obtained from UC and CC mucosa and submucosal layers. We used genomic data mining from pragmatic studies to demonstrate how biomedical studies can use the technology. By extracting new and useful biomedical and surgical sciences knowledge, we hope to develop significant momentum for applications that may have medical/surgical diagnostic potential in IBD laboratories. The genomic and proteomic patterns we noted show greater intensity in the mucosa and submucosa of CC *vs.* UC, perhaps indicative of a greater degree or different type of inflammation in the tissues underlying the layers [13,31,32]. It is possible that these differing genes may represent candidate biomarkers that could delineate the inflammatory colitides. These studies identified genes specifically involved in inflammatory responses generally over expressed in IBD and demonstrate that the colonic tissue transcriptomes obtained from UC/CC patients were quite different. The gene sets identified appear to distinguish UC from CC, and may serve as an excellent resource for professionals involved with gene expression data mining in a variety of clinical settings in IBD.

Proteomic Patterns

More recently, we have developed a proteomic approach to delineating UC *vs.* CC [12,13]. Using histologic mucosal and submucosal tissue layers for analyses, we used MALDI MS (Matrix-assisted Laser Desorption/Ionization (MALDI) Mass Spectrometry (MS)) for proteomic profiling along with bioinformatics cutting edge technologies [12,13]. We profiled surgical pathology resections of colonic mucosal and submucosal layers of patients with IBD undergoing colectomy in connection with restorative proctocolectomy (RPC) and ileal pouch-anal anastomosis (IPAA) [12,31,32]. We identified and compared protein profiles which had the necessary: 1) specificity; 2) sensitivity; 3) discrimination; and 4) predictive capacity to determine the heterogeneity of IBD [7] and we were able to delineate UC and CC molecularly. These molecular fingerprints are independent of tissue (mucosa, submucosa, or both) and appear to represent disease-specific markers. Once these markers are further tested, we can potentially develop IBD screening tools which will rely on antibodies to the protein(s) of interest (Figure 1). The distinction between UC and CC is of the utmost importance when determining candidacy for a pouch surgery [24,33,34]. Approximately 15-30% of IBD patients [12] face potential morbidity from an incorrect diagnosis with consequently inappropriate and unnecessary operative surgeries, underscoring the necessity of research efforts aimed at a more accurate diagnosis of the colitides [7,20].

Circulating Blood Biomarkers

Peripheral blood is a much more accessible source of cells that might be used to distinguish between CC and UC molecularly. Circulating peripheral blood cytokines are responsible for surveying the body for signs of disease. Cytokines may therefore serve as surrogates for disease-induced gene expression as biomarkers of disease status or severity. In an effort to attain this, we studied differences in the serum cytokine behaviors between UC and CC patients [14]. We aimed so that, if successful, such analysis could lead to an assay which could be applied as an easy, accurate, affordable, noninvasive and fast screening test [14]. However, although certain cytokines were found to differ between diseases and controls, no cytokine could clearly distinguish UC from CC [14]. An analysis of the literature has shown that although several attempts have been made to define the serum cytokines profile in IBD, the contradictory results of these studies do not indicate the possibility of finding the biomarker(s) among the serum cytokines at this time using the available technologies.

Differential Diagnosis and Treatment

These studies are highly relevant for creating a molecular differentiator between UC and CC among patients with IC. Curative gold standard treatment for UC is surgical intervention (RPC and IPAA [6,22]. Successful surgery removes the entire diseased colon while preserving bowel evacuation, continence, and fertility [24]. This is largely a result of careful patient selection combined with meticulous surgical technique, but most importantly correct diagnosis [16,22]. Clinical observations and experience suggest that it is difficult to identify patients with CC who are likely to have a successful outcome after RPC and IPAA surgery [6,16,23]. Thus, pouch surgery should be widely contraindicated for CC, but be an acceptable intervention for patients with UC and for those with IC who are likely to develop UC.

Despite the increased use of cutting-edge technologies, to date, there is no single, straight-forward explanation for the heterogeneous results of IBD. Clinicians use an inexact combined classification system, which includes clinical, endoscopy, radiologic, and histopathology findings in order to diagnose CC and UC and current approaches still require validation, and subsequently confirmation on patient outcomes in a large-scale clinical cohort. These studies are underway.

Clinical Relevance

The multilevel transcript observations by, histology, proteomics and genomics in tissue and blood suggest that the development of a molecular biometric-based tool that can complement the inexact classification system for diagnosis of UC and CC in patients with IC is still preliminary but promising. These studies could lend mechanistic insights into the role of molecular biometrics in IBD and may be utilized as biomarkers. This information will be invaluable to develop and improve diagnostic accuracy, prognostic and treatment precision tools to help clinicians to manage IBD in the clinical setting.

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