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Proteomic Patterns of Colonic Mucosal Tissues Delineate Crohn's Colitis and Ulcerative Colitis

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Abstract

Purpose—Although Crohn's colitis (CC) and ulcerative colitis (UC) share several clinical features, they have different causes, mechanisms of tissue damage, and treatment options. Therefore, the accurate diagnosis is of paramount importance in terms of medical care. The distinction between UC/CC is made on the basis of clinical, radiologic, endoscopic, and pathologic interpretations but cannot be differentiated in up to 15% of IBD patients. Correct management of this "indeterminate colitis" (IC) depends on the accuracy of future, and yet not known, destination diagnosis (CC/UC).

Experimental design—We have developed a proteomic methodology that has the potential to discriminate between UC/CC. The histologic layers of 62 confirmed UC/CC tissues were analyzed using MALDI-MS for proteomic profiling.

Results—A Support Vector Machine algorithm consisting of 25 peaks was able to differentiate spectra from CC and UC with 76.9% spectral accuracy when using a leave-20%-out cross validation. Application of the model to the entire data set resulted in accurate classification of 19/26 CC patients and 36/36 UC patients when using a 2/3 correct cutoff. A total 114 peaks were found to have Wilcoxon p-values of less than 0.05.

Conclusion/Clinical relevance—This information may provide new avenues for the development of novel personalized therapeutic targets.

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Keywords

Crohn's colitis; Ulcerative colitis; Colon tissue profiling; Proteomics; Mass Spectrometry

BACKGROUND

The inflammatory bowel disease (IBD), Crohn's colitis (CC) and ulcerative colitis (UC), affect approximately one to two of every 1000 people in developed countries.¹ These chronic inflammatory diseases result in significant morbidity and mortality.^{2,3} To date, there has been significant interest in identifying protein biomarkers that can diagnose or delineate these diseases. These include biomarkers in serum [PLGF-1 (placenta growth factor-1), IL-7 (interleukin 7), TGF β 1 (transforming growth factor), and IL-12P40 (interleukin 12P40)],⁴⁻⁹ in mucosal biopsies [Rho GDI α , pleckstrin, desmoglein, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA), VDAC, and C10orf76]^{10,11} and in feces (lactoferrin, calprotectin, PMN-elastase and S100A12).^{6,12-16} Clearly these biomarkers represent an advance in the field of IBD and have been used for clinical prognostic purposes as prognosticators for individualized treatment, evaluation of patients diagnosed with IBD, and differentiating quiescent from active IBD but have not been shown to distinguish a UC from a CC phenotype.^{4,5,14,15} Rather they reflect proteins associated with IBD intestinal inflammation in general.⁶ It is clear that there have been advances of trying to establish signatures that can aid in monitoring mucosal healing,^{17,18} predict disease etiology,^{19,20} or relapse,^{21,22} response to therapy,^{23,24} and/or use of drug levels, metabolites, and antidrug antibodies as biomarkers.²⁵⁻²⁸

Misdiagnosis is not an uncommon problem in IBD and often patients will have their original diagnosis changed²⁹ based on the final pathology report or development of another colitides phenotype in the ileal pouch,³⁰⁻³² potentially undermining the analysis. As such, a better means of diagnosis is needed to help to circumvent the problems of misdiagnosis and in appropriate treatment.

This paper is a deeper continuation of our recently published work³³ that investigates potential molecular features that could define a unique classifier between CC and UC from the inflamed mucosal colonic tissues. Previous findings from this laboratory^{34,35} prompted further sample collection resulting in an increased sample size allowing for a more robust analysis. Data were only collected from inflamed areas within the new samples and, therefore, no further analyses were carried out on uninfamed tissues.

Our efforts using proteomic approaches for discovery are aimed at identifying: a) subtype-specific markers (proteins, peptides, or antibodies in the biopsies) that may indicate a role in pathogenesis and b) (more long-term) noninvasive biomarkers or disease-specific patterns that do not involve endoscopic-based biopsies (such as body fluids). Identification of specific proteins in surgical specimens in this study is an encouraging start and an important finding with a high impact in terms of both future basic science and clinical care in IBD.

METHODS

Ethical consideration

This study was approved by the Meharry Medical College and Vanderbilt Institutional Ethical Review Board Committees (IRB file numbers: 100916AM206 and 080898) and conducted in accordance with the Second International Helsinki Declaration,³⁶ and participation in the study was voluntary.

Clinical Samples

Data were collected from CC (n=26) and UC (n=36) patient samples (total n=62) from the mucosal and submucosal colon tissues layers (Table 1). All patients included in the study were adults with definitive diagnosis of CC or UC; cases with any ambiguity were excluded. All collected data were processed simultaneously to minimize bias.

Patient Sample selection

Snap frozen (−80°C) colon samples were available from the Meharry Medical College Human Tissue Acquisition Shared Resource Core and Vanderbilt Gastrointestinal Biospecimen Repository or from the Cooperative Human Tissue Network. The diagnosis for each patient was determined based on standard clinical and pathologic features³⁷ and represented a consensus among treating physicians. A gastrointestinal pathologist blinded to clinical diagnosis confirmed the colitis diagnoses for each patient using de-identified final surgical pathology reports and hematoxylin and eosin (H&E) slides. For each sample, the mucosal and submucosal layers were analyzed.

Tissue Preparation

Tissues were analyzed using a histology-directed protein profiling^{38–40} approach in which high fidelity areas of inflamed mucosa or submucosa were targeted from intact thin tissue sections allowing for preservation of spatial integrity. In this approach, no additional sample preparation, such as cell sorting or laser capture microdissection is needed. The basic steps of the methodology are outlined in Figure 1. Briefly, two serial 12 µm thick sections of each sample were collected using a Leica CM1900 cryostat (Leica Microsystems, Bannockburn, IL) with one collected onto a gold-coated stainless steel MALDI target and the other collected on a standard microscope slide. Target plates containing sections for mass spectrometry were submerged in graded ethanol (70%, 90%, and 95%) for 30 seconds each to remove the majority of lipids and biological salts that can cause the suppression of protein ionization. The section on the microscope slide was stained with hematoxylin and eosin to allow for histological evaluation. Digital images of the stained sections were acquired using a Mirax Desk Scan (Mirax, Budapest, Hungary) digital microscope with a resolution of 0.23 µm/pixel. The photomicrographs were reviewed by a pathologist and annotated with 200 µm circles corresponding to areas of inflammation in the mucosa and submucosa. Color coding was used to designate different histological classifications (e.g. active inflammation of the mucosa, chronic inflammation of the submucosa, etc.). Typically, 15–20 areas were selected per cell type per tissue section for mass spectral analysis. The annotated histology images were exported and merged with a digital image of the MALDI target using PhotoShop (Adobe Systems Inc. San Jose, California).³⁷ Pixel coordinates of the annotations within the merged image were transferred to an acoustic robotic microspotter (LabCyte, Sunnyvale, California) using an affine transform through the use of fiducial points. Sinapinic acid matrix (20 mg/ml in 50% acetonitrile, 0.1% trifluoroacetic acid) was deposited at the desired locations on each MALDI target. A total of 78 droplets of approximately 120 pL volume were applied in 6 passes of 13 drops with adequate time for complete drying between iterations. Coordinates of these matrix spots were then transferred to a Bruker Autoflex II (Bruker Daltonics, Billerica, MA) mass spectrometer, equipped with a SmartBeam™ laser (Nd:YAG, 355 nm). Instrumental parameters were optimized for resolution at 12 kDa. Spectra were collected in linear positive ion mode as a sum of 400 laser spots from each matrix spot with movement of the laser position within the individual matrix spot after every 50 shots. Acceptance criteria were applied to each subspectrum requiring that there were peaks in the spectrum with a signal-to-noise ratio of at least 10 and a resolution of at least 300, but no greater than 3000. External mass calibration was carried out prior to data acquisition. Collected data were sorted and organized according to their tissue of origin and histology for further analysis.

Statistical Analysis

Statistical analyses were carried out on areas of active inflammation in the mucosa. Spectra for statistical comparisons were loaded into ClinProTools (Bruker Daltonics) according to their class (disease diagnosis). Due to the high heterogeneity of IBD colon tissue, spectral grouping by patient was not used. Each spectrum was therefore treated as an independent measurement. Spectra were baseline corrected using a top hat algorithm with a baseline flatness of 0.8. Null spectra and noise spectra were excluded. Peak detection was performed by manually setting the boundaries of each individual peak. Spectra were aligned to each other allowing for a 2000 ppm peak shift.

A table of Wilcoxin rank sum p-values was generated and sorted to determine the proteins that showed the greatest difference between the classes. Receiver operator curves were generated for each peak and evaluated.

A Support Vector Machine (SVM) algorithm was generated using all of the data from actively inflamed areas within the mucosa. The optimal number of peaks in the model was determined automatically and the k-nearest neighbors was set to 3. In the absence of sufficient samples of an independent validation set, the accuracy of the model for classification was estimated using a leave 20% out cross validation. In this approach, 20% of the data were randomly selected to be left out and the remaining 80% were used to build a classification model. The model was then applied to the 20% that were left out and the results evaluated. This was carried out over a total of 10 iterations with a different randomly selected 20% left out each time allowing for an overall classification accuracy to be estimated. Once the method was optimized, it was applied to classify all spectra in the data set. The results were manually interpreted to determine the percentage of spectra from each patient that classified in agreement with the pathology diagnosis.

RESULTS

Proteomic pattern profiling of colon mucosal and submucosal tissue layers using mass spectrometry technology along with sophisticated bioinformatics tools allowed for the identification of patterns within the complex proteomic profiles that discriminated between CC and UC phenotype. Due to insufficient sample size for an independent validation set, a leave- 20%-out cross-validation was used to assess the accuracy to assess the accuracy of the model.

Mucosal analysis

A total of 1257 spectra from 62 total IBD cases; 26 from CC and 36 from UC were used for statistical analysis of the mucosa. Due to the great heterogeneity of these diseases, spectral grouping by patient was not used when performing the analysis. After preprocessing, a table of Wilcoxin rank sum p-values was generated and sorted to determine proteins that could best differentiate the two diseases. Out of 312 total peaks in the averaged spectra, 114 were found to have p-values of less than 0.05.

Receiver Operator Characteristic (ROC) curves were generated for all of the peaks and evaluated. None of the peaks were found to have areas under the ROC curves of greater than 0.7.

An optimized Support Vector Machine (SVM) algorithm was developed that consisted of 25 peaks with different weighting values (Table 2) In absence of sufficient samples size to evaluate an independent dataset, a leave-20%-out cross validation was used to estimate the accuracy of the model. This resulted in an overall spectra accuracy of 76.9% with an

accuracy of 60.4% for CC and 93.3% for UC. Figure 2 shows the average spectra for CC and UC with selected peaks that are part of the SVM model indicated.

The optimized model was applied to all of the spectra and the percent correct was determined for each patient as a whole; akin to making a diagnosis for a patient. If a cutoff of 51% percent was used to determine that a patient was accurately classified, then 21/26 patients with CC and 36/36 patients with UC were correctly diagnosed. If a more stringent cutoff of 67% was used, then 19/26 CC patients and again 36/36 UC patients were correctly diagnosed. Figure 3 displays the percentage of patients with increasing spectral accuracies.

Submucosal analysis

Data were collected from the submucosal layers of the colonic tissue when available. However, after histological evaluation, it was found that only 34 total samples (19 UC and 15 CC) had active submucosal inflammation. These numbers were not sufficient for empowering statistical analyses to be carried out. As such, results from submucosal analysis are not presented here.

DISCUSSION

The use of a histology-directed mass spectral protein profiling approach allowed for high fidelity profiling of highly inflamed areas within specific layers of the colon. This technique allowed for direct targeting of areas of active or chronic inflammation within the mucosa or submucosa of thin tissue sections without the need for extensive sample preparation such as laser capture microdissection.

Examination of collected spectra along with histological evaluation of tissue sections showed a high level of heterogeneity within individual tissue sections. As such, it was not possible to use spectral grouping per patient when carrying out statistical analyses. Each spot had to be treated as an independent measurement to help to account for this variability.

A total 114 out of 312 peaks had Wilcoxin rank sum p-values of less than 0.05. This indicates that there is considerable difference in the protein expression levels between the two diseases. ROC curves were evaluated and found that none of the peaks had areas under the curve of greater than 0.7. This indicates that none of the peaks are capable of being used to independently differentiate the colitides and therefore must be used in combination for diagnosis.

The determination of a combination of peaks for diagnosis was accomplished through the generation of a Support Vector Machine algorithm consisting of 25 peaks that allowed for the successful differentiation of CC and UC. In absence of enough samples for an independent validation set, an estimation of the accuracy of the model was determined through the use of a leave-20%-out cross validation over 10 iterations resulting in 60.4% spectral accuracy for CC and 93.3% spectra accuracy for UC. The higher accuracy for UC is likely due to the considerably larger sample size for UC; 36 patients vs. 26 CC patients. It is often the case when datasets are unbalanced as in this study that the more highly represented group tends to classify better due to a more complete representation of that sample type.

The trend of higher accuracy for UC holds true when patient classification accuracies were obtained by applying the SVM model to the entire dataset. There are two different ways of considering these results. If a majority rule (>51% of the spectra correct) is used, then 21 out of 26 CC patients and all 36 UC patients were classified correctly. If a more stringent 2/3 majority is used in determining correct diagnosis, then 19 out of 26 CC patients and again all 36 UC patients were classified correctly. This results in either 92% or 89% of the patients

classified correctly when 51% or 67% of spectra correct were used as cutoffs, respectively. The classification accuracies achieved here exceed the typical histological values reported of 85%. However, in this case we were working only with well characterized samples and patients, and further work will need to be done to determine if the SVM algorithm can correctly classify patients initially diagnosed with indeterminate colitis with sufficient follow up to accurately determine clinical diagnosis.

To the best of our knowledge, this represents the second report using histology-directed MALDI MS technology to compare proteomic patterns in colonic IBD individual tissue layers, to distinguish differences between UC and CC.³³ This study expands on our pilot study of identified novel mass-to-charge discriminatory features in IBD specimens.³³ The combination of a specific proteomic evaluation of the colonic mucosal layer, and the presumed resultant detection accuracy will not only: a) create new therapeutic options to reduce relapses but will b) open new avenues for selection of the specifics of a surgical approach to prevent occurrence of dysplasia/neoplasia in the remaining rectal tissue in the anal transit zone (ATZ) after restorative proctocolectomy (RPC), and c) help avoid unnecessary surgeries.^{2,41} The information will also aid future studies in determining the biopathophysiology of these inflammatory diseases of the colon.

We sought in this study to understand the proteomic profile differences between the CC vs. UC which may lead to improved diagnostic accuracy and management of the IBD. This is important because those proteins with higher or lower abundance in one disease as compared to the other may be key paths/targets that uniquely cause or support these various forms of colitis. As the natural history, response to treatment and complications differ significantly among UC and CC, an accurate diagnosis prior to initiating medical therapy or performing a colectomy is of paramount importance to administer the appropriate evidenced personalized treatment. Restorative proctocolectomy is recommended for UC patients and for those indeterminate colitis (IC) patients who are predicted to develop UC. Nearly a third of patients with IBD who undergo RPC and ileal pouch-anal anastomosis (IPAA) surgery for “definitive UC” are subsequently found postoperatively to develop recurrent Crohn’s colitis in their ileal pouches.^{29,30,32} These individuals experience up to a 60% higher complication and functional failure rate, often requiring revision or diversion that may lead to excision of the pouch and a permanent end ileostomy, a complication both patients and surgeons want to avoid. We believe that UC *and* CC are distinct pathologies that can be distinguished using identifiable protein patterns specific to the phenotype. We even hypothesize that within IC patients, there is a 3rd unique and as yet unidentified colitis phenotype. Therefore, identifying and understanding the pathological mechanisms associated with these colitides (UC/CC) may provide diagnostic insight that will significantly improve the diagnostic accuracy for patients with these colitides as well as for those within the IC group.

Our findings, the tissue profiles of expressed protein signatures from UC and CC groups may serve as a basis to identify those proteins specific to each of the colitides, which in turn can be used to aid in diagnosis, treatment and prognosis, and to understand the pathobiology of these diseases.

SUMMARY

We have developed a set of criteria for analyzing the tissue proteome by MALDI MS profiling using mass-to-charge ratio spectra peaks corresponding to a disease phenotype, an analysis that will be used to support the diagnostic feasibility of differentiating IBD. Results from our studies analyzing colon tissue profiling have identified promising proteins using a total of 62 patient profiles that may permit accurate disease classification, diagnosis, and

prognosis. Our analyses have identified these unique protein signatures of interest found in the inflamed mucosal layer of the colon.

In conclusion, this report is updated with an increased sample size to allow a more robust analysis. Identification of these proteins is underway. After protein identification we will test our hypothesis of delineating indeterminate colitis (IC) into either UC or CC through identifying same signatures in endoscopic tissue samples from patients with colitis as well as eventually creating a serum biomarker assay to delineate the inflammatory colitides. This information may provide new avenues for the development of novel diagnostic, prognostic, and therapeutic targets.

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CLINICAL RELEVANCE

It is now well established that CC and UC represent two distinct forms of chronic inflammation and have different causes and discrete mechanisms of tissue damage. The distinction between UC and CC is currently made on the basis of clinical, radiologic, endoscopic, and pathologic assessments that are utilized to guide both medical and surgical therapy and cannot be differentiated in 15% of IBD patients. This distinction cannot always be made due to the fact that the pathological features of UC and CC often overlap in the acute phase of disease as well as in chronic situations and can lead to misdiagnosis. In addition, there are another 15% of undiagnosed IBD when the diagnosis is unclear, this condition is labeled “indeterminate colitis” (IC). The treatment options for UC and CC differ significantly. Therefore an accurate diagnosis is of paramount importance in terms of determining medical care, surgical intervention, and prognosis. About 90% of patients diagnosed with IC undergo surgery for fulminant colitis, contrasting with only 30% of patients in whom UC or CC are more confidently diagnosed. Additionally, failure to recognize characteristic signs of CC such as granulomas and transmural inflammation often leads to errors in pathological interpretation. Therefore, there is up to 15% misdiagnosis rate of CC as UC in addition to the 15% of cases being labeled as IC. To our knowledge, this laboratory is the first to undertake a novel cutting-edge study using histology-directed Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI MS) protein profiling to compare proteomic patterns in IBD of individual colonic tissue layers, in order to distinguish the differences between UC and CC. A mass spectrum consists of hundreds of peaks, each corresponding to a different protein present in the tissue section. Results from our preliminary studies analyzing colon tissue profiling, have identified promising proteomic patterns in a total of 62 patient profiles that may permit accurate disease transformation. The combination of a proteomic evaluation of the colonic mucosal layer, and the translational resultant detection accuracy will not only: a) create relevant new therapeutic options in order to maintain remissions, but will b) open promising new avenues for selection of the specifics of a personalized medical and surgical approach and c) help avoid unnecessary surgeries. The information will open future insight to understand the biopathophysiology of the colitides.

Proteomic approaches may lead to improved diagnostic accuracy in IBD as well as delineation of IBD by non-invasive, easier, more accurate and faster screening. An accurate diagnosis of IBD prior to initiating medical therapy or performing colectomy is of paramount importance in terms of personalized medical therapy, surgical intervention, and prognosis.

Figure 1.

Histology-directed mass spectral protein profiling. **A**, A 12 μm thick tissue section is placed on a gold-coated MALDI target. **B**, a serial section is mounted on microscope a slide for H&E staining. **C**, a pathologist marks areas of interest on a photomicrograph of the H&E stained section (blow up for detail) **D**, the H&E picture is merged with a picture of the MALDI target and coordinates are generated. **E**, tissue is spotted with matrix in the designated areas using a robotic spotter. **F**, spectra are generated from the matrix spots corresponding to different cell types.

Figure 2.

Average spectra from CC (blue) and UC (red). Insets show areas that encompass peaks that are part of the Support Vector Machine model, denoted by *. A total of 25 peaks were included in the SVM model, 7 of which (m/z 3409, 3651, 3729, 5655, 5696, 6695, and 6815) are highlighted here.

Figure 3. Graphical representation of the percentage of spectra classified correctly. CC patients are in blue and UC are in red. UC showed overall higher classification accuracy as noted by larger proportion of patients with a higher percentage of spectra correctly classified.

Table 1
Meta-data information of 62 subjects used in the study

The values are presented per disease and area of the colon affected. Moderate disease is categorized as patients with symptoms such as low grade fever, slight weight loss, mild abdominal pain and, tenderness and intermittent nausea or vomiting, or anemia. Severe disease is categorized as those with persistent symptoms despite conventional glucocorticoid or biologic agent therapy as outpatients, or individuals presenting with high fevers, persistent vomiting, intestinal obstruction, significant peritoneal signs, cachexia, or evidence of an abscess.

Meta-data of Subjects	Crohn's Colitis	Ulcerative Colitis
Number of subjects	26	36
Age (mean $\pm$ SD)	38.2 $\pm$ 14.3	45.3 $\pm$ 17.2
Range (years)	18–65	19–84
White	17 (65%)	32 (89%)
Black	7 (27%)	0 (0%)
Other	2 (8%)	4 (11%)
Male	12 (46%)	21 (58%)
Female	14 (54%)	15 (42%)
Moderate activity	16 (62%)	20 (56%)
Severe activity	10 (38%)	16 (44%)
COLON (Regions Analyzed)		
Ascending	16 (62%)	6 (16%)
Transverse	2 (8%)	10 (28%)
Descending	5 (19%)	17 (47%)
Cecum	3 (11%)	3 (8%)

Table 2
Peaks in the Support Vector Machine model

The masses and relative weighting of the 25 peaks in the model are given. The higher the weighting value, the more important the peak is to the model. Each mass represents a protein that helps to differentiate UC and CC.

Mass	Weight
3409	0.324
3651	0.281
3729	0.356
4228	0.347
4482	0.285
4538	0.285
4877	0.393
5316	0.305
5655	0.304
5696	0.297
6695	0.586
6815	0.345
8097	0.411
8407	0.296
8454	0.322
9165	0.460
9247	0.297
9622	0.301
9960	0.341
10063	0.295
10131	0.320
10579	0.424
12168	0.310
15084	0.292
19925	0.440