Original Article

Intestinal protein expression profile identifies inflammatory bowel disease and predicts relapse

Jun Shen1,2, Yuqi Qiao1,2, Zhihua Ran1,2, Tianrong Wang1,2, Jiangtao Xu3, Jinsun Feng3

1Division of Gastroenterology and Hepatology, Shanghai Jiao-Tong University School of Medicine Renji Hospital, Shanghai Institute of Digestive Disease; 2Key Laboratory of Gastroenterology & Hepatology, Ministry of Health (Shanghai Jiao-Tong University), Shanghai, 200127, China; 3National Engineering Center for Biochip at Shanghai, Shanghai, 201203, China

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Abstract: To date, most studies have applied individual factors as indicators of disease classification and prognosis. The aim of this study is to determine whether clustering analysis of protein expression profiles in intestinal epithelia improves classification and prognosis in patients with inflammatory bowel disease (IBD). One hundred and twenty Crohn’s disease (CD) patients, 117 ulcerative colitis (UC) patients and 120 cases of nonspecific colitis provided intestinal biopsy samples for tissue microarray (TMA). Both unsupervised and supervised analyses were used for evaluation of clustering and association with relapse. There was a significant concordance between cluster groups based on immunostaining data of TMA and clinical classification in distinguishing IBD from nonspecific colitis (kappa=0.498, p<0.001). CD27, CD70, CD40, TRAF3, TRAF4 and TRAF2 presented similar immunostaining features, which were different from clusters of CD154, CD80 and TRAF5. Moreover, higher expression of TRAF2 was a predictor of relapse in patients with UC (p=0.006). Thus, protein expression profiles can distinguish IBD and nonspecific colitis, and combination analysis protein expression profiles show that TRAF2 can predict relapse of UC.

Keywords: Crohn’s disease, ulcerative colitis, classification, relapse

Introduction

Lower GI tract inflammation can be divided into highly heterogeneous groups of diseases, and a major differential diagnosis is inflammatory bowel disease (IBD) [1]. When patients present with symptoms suggestive of IBD, combinations of invasive and non-invasive tests can be used to help distinguish nonspecific colonic inflammation from IBD, or distinguish Crohn’s disease (CD) from ulcerative colitis (UC), which are the main subforms of IBD [2]. Chronic inflammation to intestinal mucosa imparts many histologic abnormalities that may reinforce the clinical impression and narrow the differential diagnosis, which is especially important if the pathogen for the inflammation remains unclear. A correct classification of chronic inflammatory injury to ileocolonic mucosa is important for the success of both medical and surgical therapeutic strategies [3]. Despite the advent of new molecular technologies for the examination of serum proteins and genetic sequences, the diagnosis and evaluation of CD and UC based on endoscopic and histologic criteria remain unchanged [4]. More importantly, it is known that failure to achieve mucosal healing with therapy is associated with worse disease course [5]. However, the precise diagnosis of CD or UC cannot always be established with the available diagnostic tools because of overlapping features of CD and UC [6]. Although various histological patterns reflect severity and duration of IBD, few samples have specific diagnostic features [7]. Therefore, the correlation with endoscopic and clinical information is essential to receive a specific diagnosis and a fair evaluation of IBD.

Recently, there has been an increase in interests to discover new biomarkers of IBD to predict future patterns of disease and to help diagnosis, treatment, and prognosis. Most patients will probably alternate between remission and...
Intestinal protein expression and inflammatory bowel disease

Table 1. Characteristics of patients with inflammatory bowel disease

<table>
<thead>
<tr>
<th></th>
<th>CD (n=120)</th>
<th>UC (n=117)</th>
<th>NC (n=120)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at onset (yrs)</td>
<td>33.20 (30.64-35.76)</td>
<td>40.84 (37.98-43.69)***</td>
<td>34.25 (31.60-36.90)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>19.25 (18.98-19.52)***</td>
<td>19.92 (19.61-20.22)***</td>
<td>22.22 (21.82-22.63)</td>
</tr>
<tr>
<td>Gender (Female/Male)</td>
<td>48/72</td>
<td>51/66</td>
<td>46/74</td>
</tr>
<tr>
<td>Relapse (n)†</td>
<td>78</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>Extent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ileitis</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ileocolitis</td>
<td>57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colitis</td>
<td>56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prolongedsigmoiditis</td>
<td></td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Left sided colitis</td>
<td></td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>Pancolitis</td>
<td></td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>Therapy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-ASA/ SASP</td>
<td>101</td>
<td>108</td>
<td></td>
</tr>
<tr>
<td>Glucocorticoid</td>
<td>46</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>Azathioprine</td>
<td>19</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Infliximab</td>
<td>12</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

CD, Crohn’s disease; UC, ulcerative colitis; NC, nonspecific colitis; BMI, body mass index; 5-ASA, 5-aminosalicylic acid; SASP, salicylazosulfapyridine. *End point is designated as one year after remission; **Significantly different from nonspecific colitis, p<0.001.

relapse, with 10% having a relapse-free course after 10 years, and few having a continuously active course [8]. To optimize prognosis, it is important to identify prognostic factors that predict disease course at disease onset [9]. Several biomarkers have been studied in IBD as diagnostic aids, indicators of disease activity or severity, and to predict the risk of relapse in those patients in remission [10, 11]. However, none of these individual biomarkers has enough high sensitivity and specificity for accurate differential diagnosis among CD, UC and other nonspecific colitis. Individual factors as indicator of disease activity and prognosis are still conflicting, rigorous additional studies [12, 13]. Thus, panels of biomarkers are considered by clinicians for the management of IBD patients. One of the opportunities to identify and/or validate molecular signatures is provided by alternative high-throughput approaches such as tissue microarrays (TMA) [14, 15]. Immunohistochemistry on TMA may be a practical approach both in validation studies and in routine testing.

To date, most studies only apply individual factor as indicator of disease classification and prognosis, while seldom have addressed the unsupervised and supervised analysis. The aim of present study is to determine whether clustering analysis of protein expression profiles in intestinal epithelia improves classification and prognosis in patients with IBD.

Patients and methods

Patients and samples

This study comprised 120 CD patients and 117 UC patients who underwent endoscopy between Dec 2006 and Dec 2009 at Renji Hospital, Shanghai Jiao-Tong University School of Medicine. 120 cases of nonspecific colitis were obtained from Renji Hospital between Jan 2009 and Jan 2010 after informed consent. More than 600 biopsy specimens were studied using tissue microarrays. Patients with IBD were followed for one year after inducing remission, or less if they relapsed. Inclusion criteria were: clinical remission for at least 1 month at study entry as defined by a Crohn’s Disease Activity Index (CDAI) of less than 150 or Sutherland Disease Activity Index. Exclusion criteria were: pregnancy; previous small bowel resection or colostomy; use of prednisone or budesonide within 30 days of study entry; and antibiotic use at study entry. Patients receiving oral mesalamine, azathioprine or methotrexate were excluded if their medication dose had been altered within 30 days (oral mesalamine)
or within 3 months (azathioprine or methotrexate) before study entry. Ethical approval for the study was obtained from the Research Ethics Committee of Renji Hospital, Shanghai Jiao-Tong University School of Medicine.

Simple Endoscopic Score for Crohn’s Disease (SESCD) [16] and Baron score [17] were used for the endoscopic evaluation of CD and UC respectively. Patients were instructed to communicate with the research coordinator if they developed symptoms suggestive of an exacerbation, at which time a visit with a study doctor was arranged to confirm relapse.

**Tissue microarrays and immunohistochemistry**

Tissue microarrays were designed as described previously [18] by using two 0.6-mm tissue cores per case, taken from formalin-fixed, paraffin-embedded archival biopsy blocks, along with different controls, to ensure reproducibility and homogenous staining of the slides (Shanghai Biochip Co Ltd, Shanghai).

The antibody choice was empirical, based on availability and suitability for paraffin-embedded archival material. Immunohistochemistry was done using DAKO Envision™ System in the autoimmunostainer (Dako Autostainer, Copenhagen, Denmark). Primary antibodies used in this study included CD27 (1:50, NeoMarkers), CD70 (1:50, R&D), TRAF2 (1:50, Santa Cruz), TRAF3 (1:200, Santa Cruz), TRAF4

<table>
<thead>
<tr>
<th>Proteins</th>
<th>CD (quick score)</th>
<th>UC (quick score)</th>
<th>NC (quick score)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD27</td>
<td>4 (2-6)***</td>
<td>2 (1-4)</td>
<td>2 (1-4)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD70</td>
<td>2 (1-4)*</td>
<td>3 (2-6)***</td>
<td>2 (0-3)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TRAF2</td>
<td>2 (1-4)**</td>
<td>6 (4-9)***</td>
<td>3 (3-6)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TRAF3</td>
<td>1 (0-2)**</td>
<td>1 (0-1)***</td>
<td>1 (1-2)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TRAF4</td>
<td>2 (1-6)**</td>
<td>3 (2-6)***</td>
<td>3 (3-6)</td>
<td>0.0046</td>
</tr>
<tr>
<td>TRAF5</td>
<td>6 (4-8)***</td>
<td>3 (2-6)***</td>
<td>12 (6-12)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>STAT3</td>
<td>2 (0-3)</td>
<td>2 (1-3)***</td>
<td>2 (0-3)</td>
<td>0.0002</td>
</tr>
<tr>
<td>CD40</td>
<td>0 (0-1)</td>
<td>0 (0-1)</td>
<td>0 (0-1)</td>
<td>0.0852</td>
</tr>
<tr>
<td>CD154</td>
<td>8 (4-12)</td>
<td>4 (3-8)***</td>
<td>8 (8-8)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD80</td>
<td>6 (2-9)***</td>
<td>2 (1-6)***</td>
<td>8 (6-12)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*Significantly different from nonspecific colitis, p <0.05. **Significantly different from nonspecific colitis, p <0.01. ***Significantly different from nonspecific colitis, p <0.001. CD, Crohn’s disease; UC, ulcerative colitis; NC, nonspecific colitis; Mmab, Mouse monoclonal antibody; Rpab, Rabbit polyclonal antibody; Rmab, Rabbit monoclonal antibody; data were presented as median and 25%-75% percentile.

Hierarchical clustering and k-means clustering were applied to determine the classification of protein panel. Data of quick scores were reformatted as follows: -2 designated negative staining, 2 positive staining. Missing data was left blank in scored document. We used the Cluster 3.0 (average linkage, Pearson correlation) to classify CD, UC and nonspecific colitis. Results were displayed with TreeView. Distributions of protein markers and categorical variables were compared using chi-square tests. Kappa statistic was used to assess agreement in classification of cases based on expressions of biomarkers. Multiple group comparisons were applied by one-way ANOVA and followed posthoc analysis when significantly different.

Next, all variables were analyzed for their association with relapse using binary logistic regression. Time zero was defined as study entry, and patients were followed-up to relapse or to one year or the date of early withdrawal. All continuous predictors were analyzed for logistic regression. All patients were censored at one year of

**Table 2. Characteristics of antibodies and quick scores of tissue microarrays**

**Data analysis and statistical methods**

Hierarchical clustering and k-means clustering were applied to determine the classification of protein panel. Data of quick scores were reformatted as follows: -2 designated negative staining, 2 positive staining. Missing data was left blank in scored document. We used the Cluster 3.0 (average linkage, Pearson correlation) to classify CD, UC and nonspecific colitis. Results were displayed with TreeView. Distributions of protein markers and categorical variables were compared using chi-square tests. Kappa statistic was used to assess agreement in classification of cases based on expressions of biomarkers. Multiple group comparisons were applied by one-way ANOVA and followed posthoc analysis when significantly different.

Next, all variables were analyzed for their association with relapse using binary logistic regression. Time zero was defined as study entry, and patients were followed-up to relapse or to one year or the date of early withdrawal. All continuous predictors were analyzed for logistic regression. All patients were censored at one year of
follow-up, death or relapse. We performed Spearman’s rank correlation coefficient to assess the relationship among endoscopic disease activity indices and clinicopathologic characteristics.

SPSS for Windows version 13.0 (Chicago, IL) was used for statistical analysis of the data. Data are presented either with their 95% confidence intervals (95% CI) or median and 25%-75% percentile. Statistical tests were two-sided at the 5% level of significance.

Results

Different protein expression profile in patients with IBD and nonspecific colitis

A total of 120 patients with CD, 117 patients with UC and 120 patients with nonspecific colitis entered the present study. 78 patients with CD and 65 patients with UC relapse within one year of follow-up. Characteristics of enrolled subjects were shown in Table 1.

The expression of ten proteins was studied by immunohistochemistry using tissue microarrays. The quick scores of staining for all antibodies were heterogeneous among patients with CD, UC or nonspecific colitis (Table 2). Examples of staining are shown in Figure 1. Multiple comparisons indicated that quick score only showed minor significance in CD40 between patients with IBD and patients with nonspecific colitis (P=0.085, P≤0.2).

Unsupervised classification of protein expression profile

First, unsupervised hierarchical clustering analysis with average linkage was applied to the dataset of ten biomarkers. Proteins were ordered on the horizontal axis and samples were on the vertical axis based on similarity of expression profiles (Figure 2). Unsupervised hierarchical clustering analysis did not produce a dendrogram with well-defined cluster groups as CD, UC and nonspecific colitis (Figure 2A). Only a trend toward classification of IBD and nonspecific colitis was identified (Figure 2A, tree image). There was a significant concordance between cluster groups based on immunostaining data of TMA and clinical classification in distinguishing IBD from nonspecific colitis (kappa=0.498, P<0.001). Although the combined protein expression patterns in IBD cluster could hardly be subdivided to CD cluster and UC cluster because cases were scattered, we tried to figure out the trend to subgroup CD (Figure 2B) and UC (Figure 2C). However, protein expression patterns in IBD clusters showed a low concordance to subclassify UC from IBD (kappa=0.395, P<0.001). The combined protein expression patterns could not define a CD cluster (kappa=-0.051, P=0.474).

Second, in the present study, IBD cluster could not be clearly subdivided to a CD cluster and a UC cluster with hierarchical clustering analysis. Thus, we then analyzed the correlations
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between clinical classifications and expression profiles with k-means clustering analysis and chi-square tests. Similarly, no significance of overall concordance was indicated between three cluster groups based on k-means clustering analysis and clinical classification of CD, UC and nonspecific colitis (kappa=0.045, P=0.223).

Unsupervised hierarchical clustering analysis also found two major protein clusters that were clearly identified (Figure 2, above dendrogram). Despite heterogeneous expression, such analysis and color display highlighted groups of correlated proteins across correlated samples. CD27, CD70, CD40, TRAF3, TRAF4 and TRAF2 presented similar immunostaining feature, which was different from cluster of CD154, CD80 and TRAF5.

Supervised analysis of protein expression profile found factors associated with endoscopic disease activity index and relapse

Seven cases with CD were excluded from SES-CD evaluation because only small bowel disease was involved. When Spearman’s rank correlation coefficient was used to assess the relationship between endoscopic disease activity indices and protein expression profiles, no significant association was indicated between protein expression profiles and endoscopic disease activity in patients with IBD (all P>0.05).

Logistic regression exploring possible interactions among clinicopathologic variables showed that only higher expression of TRAF2 was a predictor of relapse in patients with UC (P=0.006).
Discussion

When suspecting IBD, colonoscopy with biopsy is crucial for diagnosis and evaluation. In fact, histopathological reports often mention a diagnosis of nonspecific colitis, which is hard to separate colitis with similar histological patterns but distinct distribution patterns. Although nonspecific disease states are recognized, histological patterns can reflect pathogenesis, severity and duration. Furthermore, therapeutic decisions can be directed more appropriately if endoscopy and biopsy can reliably distinguish IBD from similar symptoms caused by other inflammatory or non-inflammatory disease in intestine, or if one could distinguish CD from UC [21]. In addition, few specific pathologic markers in IBD could help monitor response in the clinic or in clinical trials. Moreover, reliable prediction of the recurrence would help appropriate therapy to those who would most likely benefit from it and avoid the excessive maintenance therapy in patients with a low potential of relapse. Thus, in the present study, protein expression profiles are used as a framework to show patterns in classification, endoscopic assessment and prognosis in IBD patients.

Protein expression profiles distinguish IBD and nonspecific colitis

The diagnostic differentiation between IBD and nonspecific colitis is sometimes difficult. Moreover, in the search for molecular markers in IBD, individual markers that are specific and sensitive enough to differentiate between CD and UC are still lack [22]. In IBD, different pathways are activated, leading to the immune intolerance of normal intestinal flora. Thousands of protein networks are involved in the pathogenicity. Thus, clinicians should consider a panel of biomarkers for the differentiation, management and follow-up of IBD patients [23]. This study reflects our concerns over mucosal biopsy assessment of colitis and its role in accurately addressing the differentiation of IBD. We find that IBD and nonspecific colitis can be distinguished by tissue microarray. However, tissue microarray of IBD cluster could not be clearly subdivided to a CD cluster and a UC cluster. Low-grade inflammation under endoscopy is often reported as nonspecific colitis, which can be confusing to clinicians. Pathologic report of inflammation may be missed without biopsy of intestinal mucosa that appears normal during endoscopy [24]. On the other hand, microscopic colitis also presents essentially normal endoscopy but with histologic inflammation of colonic mucosa [25]. Alternatively, both acceptance and ignorance of all nonspecific colitis report as being a clinically significant diagnosis may lead to inappropriate management. Increased microscopic inflammation of the intestine is also present in healthy individuals [26], which should be carefully distinguished with IBD. However, colonic CD may be difficult to distinguish from UC on endoscopy or microscopic examination of biopsy samples [27]. CD and UC have significant overlap in mucosal immunity, which leads to consider multiple ways to distinguish them such via serology and gene expression.

Another finding from the present study is that TRAFs and their associated pathways can be divided into different groups based on diverse protein expressions. Cluster designed microarrays show that similar gene expression patterns indicate similar function [28]. It has been shown that TRAFs participate in the activation of NF-κB, JNK and MAPK pathways by recruiting CD27, CD30, CD40 or CD80 pathways [29]. It is clear that TRAFs have individually specific functions or act redundantly in transmitting signals via different receptors [30]. In particular, all the biomarkers in this study can be implicated for a better understanding of the mechanism regulating canonical or non-canonical NF-κB activation [31]. Although additional studies are required to clarify the exact mechanism of clustered proteins in IBD, we suspect that the clustered proteins are potentially involved in the similar signaling cascades.

Protein expression profiles predict relapse

Assessment of disease activity in patients with CD and UC is important both in clinical practice and in clinical trials. The importance of evaluating endoscopic disease activity in the long-term management of IBD is to distinguish quiescent from active disease and to establish mucosal healing [32, 33]. Serological markers such as acute phase reactants, cytokines and adhesion molecules, and fecal markers such as calprotectin and lactoferrin have been studied for assessment of disease activity [34]. However, the correlation between the clinical indices of activity and endoscopy or histology is variable.
[35]. In our present study, it is found that protein expression profiles do not show a trend of association with endoscopic disease activity. The reason for such discrepancy is not clear yet and a subjective impression of endoscopic scoring results of IBD may be the reason. To date, in the assessment of the severity of intestinal mucosa inflammation in patients with IBD, serological and fecal markers also show controversial correlations with endoscopic disease activity [36, 37]. Although the cytokines, cytokines receptors and/or cytokine transcripts have been studied in the intestinal mucosa and have been found to be elevated correlating with endoscopic disease activity [38, 39], combination of immunomarkers in intestinal mucosa, endoscopic disease activity index and clinical activity score might be used simultaneously as assessment of disease severity in patients with IBD.

Diverse biological markers involved in the pathogenesis of IBD have been proposed as predictors of recurrence after treatment [40]. Recently, studies focusing on predicting disease relapse within the first year are emerging [41]. Several studies have indicated that higher concentration of fecal calprotectin might be a predictor of disease relapse within 12 months [42, 43]. We find that higher expression of TRAF2 is a predictor of relapse in UC patients within 12 months. By recruiting TCR-related intracellular molecules into the TRAF2 complex or regulated by costimulatory molecules, TRAF2 provides the T cell with a high level of NF-κB activity [44]. TNF-α induces the ubiquitination of TRAF2 to increase NF-κB-inducing kinase (NIK) phosphorylation. Consequently, the non-canonical pathway of NF-κB is activated [45]. However, we can’t ignore that controversy exists regarding the importance of serological, fecal and immunostaining markers in determining relapse in IBD. Preliminary data from few adequately powered prospective studies and varying definitions of relapse may be the explanations for the controversy.

Conclusions

Although more repeated studies of a longer follow-up on a larger series of IBD patients is required. Our study indicates that protein expression profiles may be a clinically useful approach to show some patterns in classification and prognosis in patients with IBD.

Acknowledgments

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Conflict of interest statement

None.

Address correspondence to: Dr. Zhi-hua Ran, Division of Gastroenterology and Hepatology, Shanghai Jiao-Tong University School of Medicine Renji Hospital, Shanghai Institute of Digestive Disease; Key Laboratory of Gastroenterology & Hepatology, Ministry of Health (Shanghai Jiao-Tong University), 1630# Dongfang Road, Shanghai 200127, P R China. Phone: +86 21 63260930; Fax: +86 21 63266027; E-mail: ranzhihua62@sina.cn

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