Evaluation of Responsive Gene Expression as a Sensitive and Specific Biomarker in Patients with Ulcerative Colitis

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Background: Clinical trials in ulcerative colitis (UC) rely on certain parameters to evaluate responses that are highly subjective or of low sensitivity. Here, using a select group of genes, we tested the accuracy of gene expression analysis as a biomarker of clinical, endoscopic, and histologic improvements.

Methods: Intestinal biopsies were obtained from UC patients included in two cohorts. Cohort 1 was used to select for genes whose expression was modulated in active (vs. inactive) UC. Cohort 2 included patients recruited in a phase II study receiving placebo, mesalazine, or dersalazine sodium for 4 weeks. The expression of 44 genes identified in Cohort 1 was assessed at weeks 0 and 4, and was then correlated with biomarkers, as well as with clinical, endoscopic, and histologic scores.

Results: Significant changes in the expression of 31 of the 44 genes tested were detected in Cohort 2 at week 4. Gene expression (ΔCt) significantly correlated with the total Mayo score, C-reactive protein (CRP), and fecal calprotectin. The number of genes significantly regulated at week 4 was highly associated with histologic and endoscopic responses. Logistic regression analysis identified four separate genes (IFITM1, ITGB2, IL1R2, IL2RA) whose relative change was independently associated with endoscopic remission with high specificity and sensitivity.

Conclusions: Change in the expression of a select set of genes can serve as an early biomarker, one with high specificity and sensitivity to clinical, endoscopic, and histologic responses. This could represent a new tool for identifying early response to treatment in mild to moderately active UC patients.

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Ulcereative colitis (UC) is a chronic remitting and relapsing inflammatory disease that affects the large intestine.1 Therapeutic options for UC patients include aminosalicylates in mild-moderate disease and corticosteroids, immunosuppressants, or biologicals for more severe cases. One-third of patients receiving corticosteroids will become steroid-dependent and one-fifth will require a colectomy within a year. In this group of steroid-resistant patients azathioprine is only modestly effective, while anti-tumor necrosis factor alpha (TNF-α) treatment achieves long-term (1 year) remission one-third of the time.2 Consequently, much effort has been devoted to identify alternative therapeutics that can effectively induce and maintain remission in patients with UC, especially those with refractory disease.

When assessing new therapeutic strategies in clinical trials it is critical that objective, quantitative, and sensitive tools are employed in order to evaluate clinical activity and response to treatment at early stages. Disease activity in UC patients is determined by an assessment of clinical symptoms and endoscopic lesions. Most clinical trials also record serological and fecal biomarkers, although these have low diagnostic accuracy for the detection of ongoing inflammation, particularly in cases of mild or moderately active UC.3 Clinical symptoms such as diarrhea and pain are highly subjective, and correlations with the presence of endoscopic lesions are usually poor.4 Nonetheless, healing of the mucosa is associated with long-lasting remission and a reduced need for surgery in patients with UC.5,6 While the need for endoscopic monitoring of patients is well accepted, this procedure relies on a highly subjective and narrow score to determine the presence of lesions.

Different studies have used gene expression to evaluate disease activity or to predict response to treatment.7,8 These studies have shown that dramatic changes occur in the transcriptional signatures of the inflamed intestinal mucosa, suggesting that the
expression patterns of certain genes could constitute valid biomarkers of disease activity. Here we examined the effectiveness of transcriptionally profiling intestinal biopsies in order to find sensitive biomarkers of disease activity. Our specific aim, therefore, was to identify a set of genes whose expression is regulated in the intestinal mucosa of active UC patients and that changes significantly during disease remission or recurrent inflammation, and histologic standpoint. To validate this signature we used samples from subjects participating in a multicenter, double-blind, randomized, placebo and mesalazine controlled phase II study, one designed to test the efficacy of dersalazine sodium in mild to moderate UC patients. Dersalazine sodium, a new chemical entity with antiinflammatory properties, may provide a valuable therapeutic alternative to corticosteroids, immunosuppressants, and anti-TNF-α antibodies. Dersalazine sodium combines a potent platelet-activating factor (PAF) antagonist and 5-aminosalicylate (5-ASA) and it is carried unabsorbed through the intestinal tract to deliver the anti-PAF moiety and 5-ASA. Ultimately, our study assessed the use of specific gene expression profiling as a standardized, objective, and highly sensitive tool for monitoring the efficacy of new therapies in mild to moderately active UC patients.

**MATERIALS AND METHODS**

**Patients Enrolled**

This study was conducted in two phases that used samples from two independent cohorts of patients.

Gene selection phase (Cohort 1): Cohort 1 included non-IBD controls (n = 4), patients with UC in remission (n = 13), and patients with active UC of varying severity (n = 10). In this phase we tested changes in the expression of 88 genes selected from arrays performed in-house using biopsies from active UC or controls (Clinical set), from expression assays performed in an experimental model of colitis (Palau set), from the literature or from publicly available data-sets (GO). We chose genes that exhibited at least a 2-fold change in expression in active UC compared to uninflamed mucosa (Table, Supplemental Digital Content 1, http://links.lww.com/IBD/A44). Genes selected in this phase included well-described proinflammatory pathways. Clinical and demographic characteristics of patients in Cohort 1 are shown in Table, Supplemental Digital Content 2, http://links.lww.com/IBD/A45.

Gene validation phase (Cohort 2): Cohort 2 included a subset of patients (n = 28) with active, mild-to-moderate UC who had been enrolled in a randomized, double-blind, parallel group, double-placebo, and active reference controlled multicenter study (http://clinicaltrials.gov/ct2/show/NCT00808977?term=dersalazine&rank=1). Patients were recruited at 10 independent sites from 4 European countries (Spain, Belgium, Hungary, and Slovakia).

Patients were randomized into the following treatment groups: placebo (n = 9; 6 males; mean age 41.8 ± 11.9), mesalazine (n = 7; 3 males; mean age 43.8 ± 14.3), and dersalazine sodium (n = 12; 6 males; mean age 42.5 ± 14.0). The total Mayo score (0–12), endoscopic Mayo subscore (0–3), histologic score (0–5), serum C-reactive protein (CRP) levels, and fecal IL-1 and calprotectin were recorded at pretreatment and at week 4. Gene expression analysis was performed from biopsies obtained at pretreatment and at week 4. Table 1 shows the clinical characteristics of patients in Cohort 2.

As previously described, clinical response was defined as a decrease from baseline in the total Mayo score of at least 3 points, with an accompanying decrease in the subscore for rectal bleeding of at least 1 point. Complete remission was defined as a total Mayo score at week 4 of 2 points or lower, with no individual subscore exceeding 1. Endoscopic or histologic response was defined as a decrease in the respective score of 1 or more at week 4.

**Sample Collection and RNA Isolation**

Biopsies from patients in Cohort 1 were obtained from the rectum or sigmoid colon during routine exploratory endoscopy. For patients in Cohort 2 (dersalazine study) colonic biopsies were obtained at screening (pretreatment visit) and at day 28 of the study. All biopsies were harvested in RNAlater (Ambion, Applied Biosystems, Spain) kept at 4°C for at least 24 hours, and then stored at –80°C. Total RNA was isolated using an RNeasyMini Kit (Qiagen, Spain). The integrity and quantity of RNA was determined with a 2100 Bioanalyzer (Agilent, Germany) and a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, USA).

**Real-time Polymerase Chain Reaction (PCR)**

Total RNA (1 μg) was transcribed to cDNA using reverse transciptase (High Capacity cDNA Archive RT kit, Applied Biosys-tems). Real-time PCR was run using an Applied Biosystems custom-designed TLDA and TaqMan Universa PCR Master Mix (Applied Biosystems). PCRs were performed using an Applied Biosystems 7900HT sequence detection system. DeltaCt (ΔCt = Ct reference gene-Ct target gene) were calculated using three endogenous control genes, ACTB (β-actin), GAPDH (glyceraldehyde 3-phosphate dehydrogenase), and UBC (ubiquitin), which were selected from the included reference genes using the Vandesompele method.

**Fecal IL1β and Calprotectin Determination**

Fecal samples from Cohort 2 patients were frozen and transferred to the central laboratory (CERBA, Sabadell, Spain) where IL-1β and calprotectin were analyzed using validated routine analytical methods.

**Statistical Analysis**

Analysis of variance (one-way ANOVA) was performed to identify those genes differentially expressed among the various groups during the selection phase. One-tailed paired t-test analysis was employed to compare gene expression at screening and at week 4 of treatment for each patient in Cohort 2. A test for correlation between paired samples, based on Pearson’s product moment correlation coefficient, was used to estimate any associations between gene expression and clinical scores and biomarkers used in the evaluation of disease activity. An unsupervised cluster analysis for ΔCt values in the responding gene set was performed using the Euclidean distance and complete linkage method. Any P < 0.05 was considered significant.
To determine the sensitivity and specificity of gene expression changes vis-à-vis clinical, endoscopic, or histologic response, we calculated receiver operating characteristic (ROC) curves and measured the area under the curve (AUC) using IBM SPSS Statistics software v. 19.0.0 (Chicago, IL). The cutoff point was identified as that with the shorter distance from the ROC curve and the random chance line (distance $= \sqrt{(1 -$ \text{specificity})^2 + (1 -$ \text{sensitivity})^2}$). To identify those genes that independently associated with endoscopic remission in Cohort 2 patients, we performed a logistic regression using IBM SPSS Statistics software v. 19.0.0.

**Ethical Considerations**

All patients in Cohort 1 were enrolled at the Department of Gastroenterology, Hospital Clinic (Barcelona, Spain) and had signed an informed consent. This study was approved by the Hospital Clinic Ethics Committee. The clinical phase IIa trial (Cohort 2) was approved by the respective Ethics Committees at each enrolling institution. All patients received oral and written information before entering the trial.

**RESULTS**

**Identification of a Transcriptional Signature in the Intestinal Mucosa of Active UC (Gene Selection Phase)**

The selection phase used genes identified in published microarrays (http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSE9452) and also relied on preclinical studies that tested dersalazine sodium in a trinitrobenzene sulfonic acid...
patients we obtained colonic biopsies from a group of 28 patients participating in a multicenter phase II study designed to examine the safety and activity of dersalazine sodium (Cohort 2). All recruited patients had a total Mayo score at screening of 5 or higher (8.0 ± 1.57; mean ± SD) and an endoscopic score of 2 or higher (2.25 ± 0.44; mean ± SD). At week 4 the average total Mayo score was 5.44 ± 3.02 (mean ± SD) and the endoscopic score 1.67 ± 0.77 (mean ± SD) (Table 1).

In our subset of 28 patients, clinical response at week 4 was achieved in 12 patients (42.8%) and complete remission in four (14.2%) (see Materials and Methods for definitions of responses). Endoscopic response was reported in 13 patients (46.4%) and histologic response in nine (32.1%) (Table 1).

Changes in gene expression between pretreatment and week 4 were also evaluated for each patient. We identified a group of 31 genes whose expression was significantly different (paired t-test P < 0.05) between pretreatment and week 4, regardless of the treatment received and the response to it (Fig., Supplemental Digital Content 3, http://links.lww.com/IBD/A46). We will refer to this set of genes as the Responsive Gene Set (RGS). To quantify the degree of gene response for each individual patient we calculated the percentage of genes in the RGS (%RGS) whose expression was significantly regulated at week 4 compared to pretreatment, with a fold change of at least 1.5 at week 4 (paired t-test P < 0.05). The %RGS is shown in Table 1 and varies significantly among patients (5.5-87.4 range).

### Absolute Expression of Selected Genes Correlates with Disease Activity

We assessed whether the absolute gene expression (ΔCt) of the selected genes at a given timepoint correlated with the total Mayo score, CRP, and/or fecal biomarkers (calprotectin and IL-1β) (Table, Supplemental Digital Content 4, http://links.lww.com/IBD/A47). Of all the parameters studied, the total Mayo score (which includes a subscore for endoscopic activity) displayed the best correlation, followed by fecal calprotectin and CRP, with expression of the majority of genes tested (Table, Supplemental Digital Content 4, http://links.lww.com/IBD/A47). Nonetheless, CRP and fecal calprotectin levels tended to be increased in patients with the highest expression of selected genes (showing good specificity), and were not able to discriminate patients with lower gene expression levels (low sensitivity) (Fig. 2 shows the correlation with one representative gene). In contrast, fecal IL-1β, at least in our cohort of patients, did not correlate either with the expression of any of the genes tested (Table, Supplemental Digital Content 4, http://links.lww.com/IBD/A47), or with the CRP values or total Mayo scores (CRP: r = 0.1; P = 0.484; Mayo score: r = −0.085; P = 0.536).

Of note, the expression at baseline of two of the studied genes, ICAM-1 and IFITM1, differed significantly between female and male patients (t-test P = 0.0357 and 0.0403, respectively). Expression of an additional three genes significantly correlated with the patient’s age: CD11D (Pearson’s correlation r = −0.44, P = 0.016), IL2RA (r = −0.43, P = 0.019), and PLA2G7 (r = −0.42, P = 0.022). Despite
these baseline differences, the expression of both genes correlated significantly with the Mayo score, both in male and female patients (not shown). This suggests not only that they are associated with disease activity independently of gender, but also that they may potentially be used as predictors of response to treatment.

To explore the efficacy of gene expression analysis to distinguish active from remitting disease, we performed an unsupervised cluster analysis of the ΔCt values for the RGS (including all 31 genes) in all available samples from Cohort 2. Figure 3 shows the distribution of samples into two large clusters. Samples with an active UC transcriptional profile were grouped into the left-side cluster, while all samples with a remitting UC profile were clustered to the right. For each individual sample, endoscopic and histologic scores are notated in the colored line at the top of the heatmap. Patients with histologic or endoscopic scores of 0–1 were clearly segregated from those with high endoscopic (3) or histologic (4–5) scores. Interestingly, patients with intermediate endoscopic (2) or histologic (2 or 3) scores are scattered throughout both clusters. This would suggest that, at least in some patients with intermediate endoscopic and/or histologic scores suggestive of mild or recovering disease, changes in gene expression are already apparent. Since these data were obtained just 4 weeks after starting treatment, it remains unclear whether these early changes in gene expression, especially in those patients with persistently high endoscopic or histologic scores, were associated with response to treatment at a later timepoint.

**Changes in Gene Expression Are Associated with Clinical, Endoscopic, and Histologic Improvements**

We next wanted to assess whether changes in gene expression over time mirrored clinical, endoscopic, and histologic changes in Cohort 2. Figure 4A shows the percentage of genes regulated in the RGS in patients grouped as clinical, endoscopic,
or histologic responders versus nonresponders. For all three criteria of disease activity evaluation, the percentage of regulated genes was significantly higher in responding versus nonresponding patients.

The percentage of change in CRP values between pretreatment and week 4 was also significantly higher ($P = 0.023$) in histologically responding versus nonresponding patients. However, the differences when comparing clinically or endoscopically responding patients did not reach statistical significance (Fig. 4B).

To test whether changes in gene expression can be reliably associated with a particular therapeutic response we calculated ROC curves, taking into account the number of genes in the RGS that showed a significant change as well as each of the response variables. The accuracy of a measure of the number of response genes associated with a histologic response proved high, with an AUC of 0.901, a sensitivity of 0.880, and a specificity of 0.833 using a cutoff point of 50% (50% of the genes showed significant changes). Similarly, the set of responsive genes was also associated with high accuracy, both to endoscopic response (AUC 0.867, sensitivity 0.84, specificity 0.93) and to clinical response (AUC 0.79, sensitivity 0.75, specificity 0.81).

Next, to narrow down the number of genes required to predict response, we performed a binary logistic regression using endoscopic remission (endoscopic score at week 4 ≤ 1) as the dependent variable, and the change in expression between week 0 and week 4 ($\Delta$Ct) for all 31 genes included in the RGS as covariables. Among the various measures of drug efficacy we chose endoscopic remission due to the objectivity it affords, as opposed to clinical remission, the subjectivity of which can be affected by a patient’s symptoms and/or a physician’s global assessment. Moreover, endoscopic remission has been precisely defined and has a documented prognostic value, which is not the case for histologic remission. Lastly, the number of patients who achieved, or failed to achieve, endoscopic remission remained balanced. Using this approach we identified four genes (IFITM1, ITGB2, IL1R2, and IL2RA) as independent biomarkers of endoscopic response, with the following coefficients: $\beta_0$ (constant) = $-95.643$; $\beta_1$ (IFITM1) = $125.061$; $\beta_2$ (IL1R2) = $-44.116$; $\beta_3$ (IL2RA) = $-45.119$; $\beta_4$ (ITGB2) = $72.608$. The calculated risk factor facilitated a 100% accurate prediction rate of endoscopic remission (cutoff value = 0.5).

**Analysis of Gene Expression Response Could Be Useful When Comparing Different Treatment Strategies**

Finally, we explored whether treatment per se could influence gene expression independently of disease improvement. We compared gene expression profiles between pretreatment and week 4 for each treatment group using a one-tailed paired analysis (Table, Supplemental Digital Content 5, http://links.lww.com/IBD/A48). As shown in Figure 5, the majority of genes regulated in the placebo or mesalazine group were also significantly changed in darsalazine-receiving patients. In addition, we identified a large number of genes (17) that were significantly regulated only in the darsalazine-receiving group. These results would suggest that while analysis of the RGS provides an effective evaluation of disease activity, independently of the type of treatment, this tool may also facilitate the identification of genes that are more sensitive to specific treatments.

**DISCUSSION**

The majority of clinical studies to date have relied, at least in part, on the amelioration of clinical symptoms as an endpoint to evaluate the efficacy of a given treatment.

The subjectivity of clinical measures and the high rate of placebo clinical response, however, make clinical assessment an unreliable tool to obtain early evidence of response. Moreover, a significant percentage of patients show endoscopic activity despite clinical response or even remission. In addition, achieving mucosal healing early during response to treatment has been shown to be a critical indicator of long-term remission. The use of imaging techniques to evaluate the presence and severity of mucosal lesions is therefore required to correctly identify active patients. Nonetheless, endoscopic scores can be highly subjective and sometimes difficult to compare among centers in multicenter trials.

Besides clinical and endoscopic scores, serological and fecal biomarkers are widely used to evaluate disease activity. Such biomarkers have the advantage, in comparison to endoscopy, of not

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**FIGURE 3.** Unsupervised cluster analysis of ΔCt values for the responding gene set (31 genes) in all samples included in Cohort 2. Samples are clustered into two groups according to their gene expression pattern. For each patient samples at weeks 1 (V1) and 4 (V6) were available. Histologic and endoscopic scores are shown at the top of the heatmap for each individual sample. For histologic scores, green represents a score of 0–1, purple a score of 2, blue 3, and red 4–5. For endoscopic scores, green represents a score of 0–1, blue a score of 2, and red a score of 3.
being invasive. Nonetheless, they do not always correlate with the presence of endoscopic lesions, showing low sensitivity in patients with mild to moderate disease. Although histologic improvement of the intestine is a good predictor of disease outcome, relying solely on this measure to determine response to treatment during early timepoints may provide low sensitivity. In some patients histologic response may be delayed and represent only the late stages of recovery.

FIGURE 4. Changes in gene expression and CRP values based on response to treatment. (A) The percentage of genes significantly regulated in the RGS was measured for each individual in Cohort 2 as follows: the percent of genes from the selected response set that were significantly regulated ($P < 0.05$, paired t-test) at week 4 compared to expression at pretreatment. (B) The percentages of change in CRP values at week 4 compared to pretreatment for each individual in Cohort 2 are also shown. The percentage of responding genes, or the percentage of change in CRP, for each individual is represented according to their clinical, histologic, or endoscopic responses. The $P$-value for the statistical analysis between means is shown for each plot (Mann-Whitney analysis).
Based on this, we decided to explore the potential of transcriptional analysis of a selected group of genes as a quantitative and objective marker of mucosal healing in response to treatment. The group of genes whose expression was shown to correlate well with disease activity in Cohort 1 (selection phase) included cytokines and chemokines, cytokine receptors, immunoglobulins, metalloproteases, antimicrobial peptides, adhesion molecules, complement proteins, PAF-related molecules, cell proliferation proteins, and proinflammatory enzymes, among others. Most of these genes had previously been shown to be regulated in active UC.8,11–14

In order to investigate the usefulness of this set of transcriptional biomarkers as predictors of disease improvement, we determined their expression in a group of UC patients participating in a phase II clinical trial (Cohort 2). The advantage of this approach is that we combined the information provided by conventional and well-established scores of disease activity with transcriptional analysis of biopsies obtained both at pretreatment and at 4 weeks after randomization. Having biopsies at both timepoints allowed not only for the analysis of absolute gene expression at a given timepoint, but also for an assessment of the relative changes that occurred during response to treatment in each individual patient. According to our study, the absolute gene expression value (ΔCt) for most selected genes (responsive gene set: RGS) significantly correlated with changes in clinical activity as assessed by the total Mayo score, which includes a subscore for endoscopic activity. On the other hand, while the correlation of biomarkers, such as serological CRP and calprotectin with ΔCt values from selected genes was lower, it was still significant for the majority of genes studied. Therefore, we can conclude that expression of a select group of genes could be employed as a sensitive biomarker of disease activity in UC patients and, at least in our cohort of patients, shows a higher degree of sensitivity than CRP, fecal IL-1β, or calprotectin.

To evaluate the usefulness of gene expression analysis to predict actual clinical, endoscopic, or histologic responses in patients, we calculated the percentage of change in our previously validated RGS. Despite the short duration of our study (4 weeks), the percentage of significantly regulated genes was significantly higher in patients with reported clinical, endoscopic, and/or histologic responses compared to nonresponders. Importantly, our analysis also revealed that the evaluation of changes in the RGS could be accurately associated with clinical, endoscopic, and histologic changes, with a sensitivity greater than 0.75 and a specificity exceeding 0.8. Importantly, we were able to identify a reduced set of genes (IFITM1, ITGB2, IL1R2, and IL2RA) whose change in expression over the 4-week treatment period independently and accurately associated with mucosal healing. This would suggest that evaluation of a therapeutic response, based on relative changes in RGS expression or on the identified reduced gene set at early timepoints (in this case, 4 weeks), may provide an objective measurement of the therapeutic effect of a given drug. Importantly, this approach overcomes the subjectivity of histologic, endoscopic, and/or clinical evaluations.

In summary, we identify a set of genes whose expression significantly correlates with disease activity. Moreover, changes in the expression of these genes were associated with mucosal and clinical improvement with high sensitivity and specificity. These changes in gene expression occur independently of the treatment administered and can be observed at earlier timepoints during treatment response. We propose the use of these biomarkers in clinical trials will contribute, in an objective manner, to the assessment of disease activity, and could serve as an effective and complementary tool to clinical and endoscopic evaluations.

**FIGURE 5.** Effect of treatment on the regulation of the responding gene set (RGS). Venn diagram (A) and table (B) showing the number of genes in the RGS significantly regulated in the placebo, mesalazine, and dersalazine treatment groups, independently of response to treatment. Genes that are common to two of the treatments, or to all three, are shown in the intersections of the diagram.
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