ORIGINAL PAPER

RJME
Romanian Journal of Morphology & Embryology
http://www.rjme.ro/

Gene expression profile of endoscopically active and inactive ulcerative colitis: preliminary data

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Abstract

Aim: Multiple cytokines and chemokines related to immune response, apoptosis and inflammation have been identified as molecules implicated in ulcerative colitis (UC) pathogenesis. The aim of this study was to identify the differences at gene expression level of a panel of candidate genes in mucosa from patients with active UC (UCA), patients in remission (UCR), and normal controls. *Patients, Materials and Methods*: Eleven individuals were enrolled in the study: eight UC patients (four with active lesions, four with mucosal healing) and three controls without inflammatory bowel disease (IBD) seen on endoscopy. All the individuals underwent mucosal biopsy during colonoscopy. Gene expression profile was evaluated by polymerase chain reaction (PCR) array, investigating 84 genes implicated in apoptosis, inflammation, immune response, cellular adhesion, tissue remodeling and mucous secretion. *Results*: Seventeen and three genes out of 84 were found significantly differentially expressed in UCA and UCR compared to controls, respectively. In particular, *REG1A* and *CHI3L1* genes reported an up-regulation in UCA with a fold difference above 200. In UCR patients, the levels of *CASP1*, *LYZ* and *ISG15* were different compared to controls. However, since a significant up-regulation of both *CASP1* and *LYZ* was observed also in the UCA group, only *ISG15* levels remained associated to the remission state. *Conclusions*: *ISG15*, that plays a key role in the innate immune response, seemed to be specifically associated to the UC remission state. These preliminary data represent a starting point for defining the gene profile of UC in different stages in Romanian population. Identification of genes implicated in UC pathogenesis could be useful to select new therapeutic targets.

Keywords: ulcerative colitis, gene expression, PCR array, *REG1A*, *CHI3L1*, *ISG15*.

Introduction

The etiology of inflammatory bowel diseases (IBDs), mainly represented by ulcerative colitis (UC) and Crohn's disease (CD) is yet to be determined. Modern hypotheses state that in a genetically predisposed host, exposure to environmental factors and altered intestinal microbiota can lead to an abnormal immune response. Endoscopic lesions are the result of polymorphonuclear activation leading to disruptions in the mucosa and possibly crypt abscess formation, corresponding to various aspects like: erythema, decreased to absent vascular pattern and erosions or ulcerations [1]. Over the past 20 years, several small genetic association studies and genome-wide association studies (GWAS) have been conducted, identifying more than two hundred risk loci for IBD implicating biological mechanisms such as autophagy, barrier defense and T-cell differentiation signaling [2]. However, the identification of genetic variants *per se* cannot explain the complex mechanisms associated with these diseases and there is the need for complementary molecular approaches, such as gene expression evaluation.

A number of gene expression studies performed in IBD, both in tissue and peripheral blood, have provided significant information towards systemic and local inflammatory response. In 2007, Wu *et al.* conducted a global gene expression study comparing colonic biopsies of CD and UC patients with infectious colitis patients and healthy control subjects, indicating that distinctive pathways contribute to disease mechanisms, with the UC gene pattern dominated by loss of epithelial homeostasis (metabolism, biosynthesis and electrolyte transport) [3].

Later, another study integrated both gene expression profiling in biopsies of UC with genome-wide genotype analyses in order to identify and prioritize the gene sets and molecular pathways most consistently associated with UC, finding 87 pathways up-regulated and eight down-regulated. Among these pathways, the most significant were related to cytokines, chemokines, and their receptor signaling. The signaling pathways more frequently up-regulated were through T-cell antigen receptor, Janus kinase/signal transducer and activator of transcription (JAK-STAT) and nucleotide oligomerization domain (NOD)-like receptor signaling [4].

A previous Romanian study in IBD patients found high expression levels of IL-8, chemokines SCYA3/4 and gluthatione S-tranferase P1 (*GSTP1*) gene in inflamed

tissue and an alteration of IL-15 and SCYA85 levels in uninflamed tissue from UC biopsies [5].

The aim of our study is to obtain preliminary data regarding the molecular signature of endoscopically active and inactive UC in a small cohort of Romanian patients. Comparison of the UC patients' gene expression profile with the profile of healthy control mucosa revealed possible molecular markers of activity/remission through correlations with the available knowledge regarding disease activity by endoscopic and histological scores.

Patients, Materials and Methods

Study population

Eight adult patients with a diagnosis of UC, either active or in remission, as well as three controls (without IBD), evaluated in the Departments of Gastroenterology and Hepatology of two academic hospitals in Bucharest, Romania – "Fundeni Clinical Institute and "Elias" Emergency University Hospital – were enrolled in the study. The three controls (two males and one female of 26, 33 and 50 years, respectively) underwent colonoscopy for screening. All patients and controls were of Romanian origin. The diagnosis was made on clinical, endoscopic and histological criteria according to the *European Crohn's and Colitis Organization* Guidelines [6] and the ongoing treatment was noted. Written informed consent was obtained from all participants prior to biopsy collection. The study protocol was approved by the local ethics committee. Biopsy samples were obtained during colonoscopy from active inflamed areas (UCA), from previously active and now in remission areas (UCR) and controls.

Score descriptions

The disease extension was defined using *Montreal Classification* where: E1: proctitis, E2: left-side colitis, E3: pancolitis [7].

The disease severity was assessed using *Partial Mayo scoring index* with values ranging between "0" and "9" [8].

The severity of endoscopic lesions was defined using the Mayo endoscopic sub-score that takes into account erosions/ulcerations, mucosal erythema, vascular pattern visibility and bleeding provoked/spontaneous, with scores ranging from 0 to 3 and with mucosal healing defined as Mayo score 0 or 1 [6, 9].

For the histological evaluation, we chose the Nancy score, which has parameters like the presence of ulceration, acute and chronic inflammatory infiltrate and defines severity on a five level scale ranging from 0 (absence of significant histological activity) to 4 (severe histological activity) [10, 11].

RNA isolation and cDNA preparation

Total RNA isolation from tissues preserved in RNA later was performed using miRNeasy mini Kit (Qiagen) according to the manufacturer's protocols. The concentration of RNA was quantified using the Nanodrop 2000 (Thermo Scientific) by measuring the absorbance at 260 nm. Moreover, the OD260/230 and OD260/280 ratios were determined to assess RNA purity. Both 260/280 nm and 260/230 nm parameters were >1.9. Next, 600 ng of RNA were reverse transcribed to cDNA. The cDNA was synthesized using the RT^2 First Strand Kit (Qiagen) following the manufacturer's instructions.

RT^2 Profiler polymerase chain reaction (PCR) Array

The Human Crohn's Disease RT^2 Profiler PCR Array (PAHS 169Z, Qiagen) was used to assess the expression of 84 key genes differentially expressed during IBD according to the manufacturer's protocol. One hundred and two µL of cDNA were mixed with 2× RT^2 SYBR Green Mastermix and RNase-free water to obtain a total volume of 2700 µL. Subsequently, 25 µL of the mix were placed into each well of the 96-well PCR array. The three steps of the cycling program were 95°C for 10 minutes for one cycle, followed by 40 cycles of 95°C for 15 seconds and 60°C for one minute. This process was performed on the ABI-7500 fast instrument (Applied Biosystems). The expression levels of each gene were normalized on the geometric mean values of five housekeeping genes (*ACTB*, *B2M*, *GAPDH*, *HPRT1*, and *RPLP0*).

Statistical analysis

Categorical variables were tested by means of the *chi*-square test, continuous variables with the Student's *t*-test and the correlations evaluated with the Pearson's test, using the Statistical Package for Social Sciences ver. 17.0 (SPSS Inc.). For data gene expression analysis, the RT^2 Profiler PCR Array software package was used. This package uses $\Delta\Delta CT$-based fold change calculations and the Student's *t*-test to calculate two-tail, equal variance *p*-values.

Results

Patients were divided into two groups based on disease severity: active and remission. Clinical and phenotypic data of the patients are summarized in Table 1. No difference in age and gender was observed between the groups of the three controls and the eight patients (average of age in controls: 36.33±12.34, average of age in patients: 43.88±15.76, p=0.479; gender, p=0.782, X^2=0.076).

The two groups of patients were homogenous for age (UCR: 46.25±21.23, UCA: 41.5±10.66, p=0.703), gender (p=0.102, X^2=2.66) and duration of illness (UCR: 7.2±6 years and UCA: 2.25±2.6, p=0.178).

The average of Mayo and Nancy scores was different between the groups (Mayo UCA: 2.25±0.5, UCR: 0.5± 0.577, p=0.004; Nancy UCA: 3±0.816, UCR: 0.5±0.577, p=0.002) and endoscopic severity (Mayo score) correlated with histological severity (Nancy score) (p<0.0001, r=0.973).

Seven out of eight patients were in treatment with 5-Aminosalicilic acid (5-ASA) and/or Azathioprine (AZA) and/or Adalimumab (ADA) when included in the study.

In the UCR group, there were two patients with "Mayo 1" endoscopic score and other two with "Mayo 0". These aspects perfectly correlated with Nancy histological assessment, with 0 points meaning colonic mucosa with minimal interstitial inflammation and hyperemia (Figure 1a) for all patients with "Mayo 0" score and 1 point, meaning

colonic mucosa with crypt distortion and moderate to severe increase of the lamina propria inflammatory infiltrate (Figure 1b) for all patients with "Mayo 1" score.

In the UCA group, three patients had mild clinical activity and one patient had severe symptoms. In this group also, there was a good correlation of clinical symptoms with endoscopy and histology. Thus, patients with "Mayo 2" endoscopic score had "Nancy 2" histological score in one case, meaning colonic mucosa with crypt distortion, dense inflammation in the lamina propria, including neutrophils and cryptitis (Figure 1c) and "Nancy 3" score in three patients, presenting colonic mucosa with multiple cryptitis and crypt abscess (Figure 1d). The only one patient with severe symptoms and endoscopic aspect ("Mayo 3" endoscopic score) had "Nancy 4" histological score, showing chronic active colitis with severe mucosal inflammation, cryptitis, crypt abscess, crypt destruction and erosion, with superficial purulent exudate (Figure 1e).

Among the 84 genes investigated, 60 genes were differentially expressed in terms of fold regulation (2<FR <-2) in UCA (57 up-regulated and three down-regulated) and 37 in UCR (36 up-regulated and one down-regulated) compared to controls (Figure 2).

Table 1 – ***Socio-demographical, clinical and morphological characteristics of patients***

Group type	**Group A (active disease – UCA)**				**Group B (remission disease – UCR)**			
Patient No.	***P 1***	***P 2***	***P 3***	***P 4***	***P 5***	***P 6***	***P 7***	***P 8***
Age at diagnosis [years]	29	55	42	40	62	22	35	66
Gender	M	M	M	M	M	F	F	M
Disease duration [years]	2	0.5	6	<1	2	3	15	9
Extension of the disease	E3	E2	E3	E2	E2	E3	E2	E2
Severity of flare (partial Mayo score)	3	7	3	3	1	1	2	2
Mayo endoscopic score	2	3	2	2	0	0	1	1
Nancy histological score	3	4	3	2	0	0	1	1
Medication	AZA	No treatment	5-ASA	5-ASA	5-ASA	AZA	ADA + 5-ASA	AZA + 5-ASA

UCA: Active ulcerative colitis; UCR: Remission ulcerative colitis; P: Patient; M: Male; F: Female; Montreal Classification: E2 – left-side colitis, E3 – pancolitis; AZA: Azathioprine; 5-ASA: 5-Aminosalicilic acid; ADA: Adalimumab.

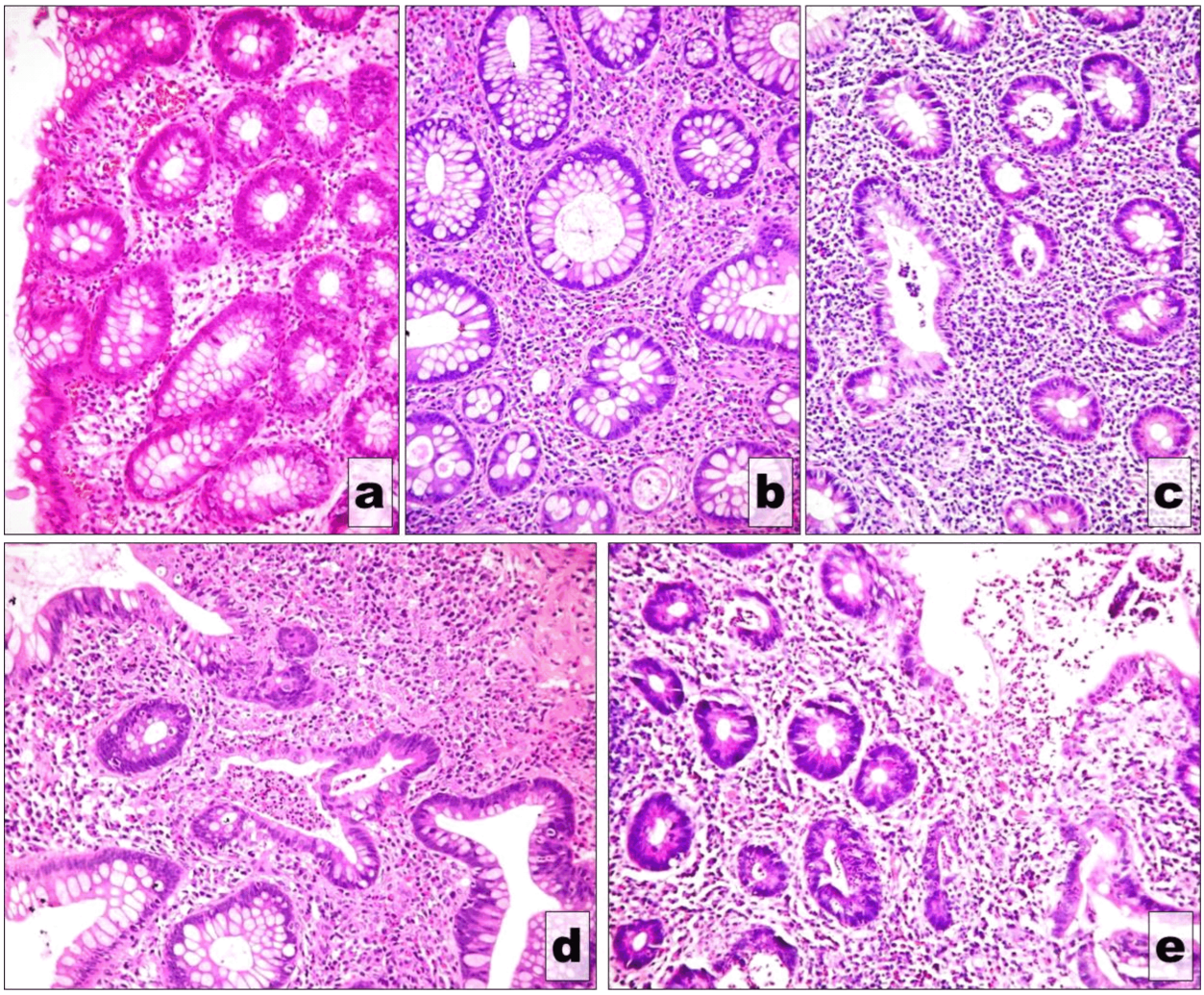

Figure 1 – ***Nancy histological score: (a) Grade 0; (b) Grade 1; (c) Grade 2; (d) Grade 3; (e) Grade 4. Hematoxylin–Eosin (HE) staining, ×200.***

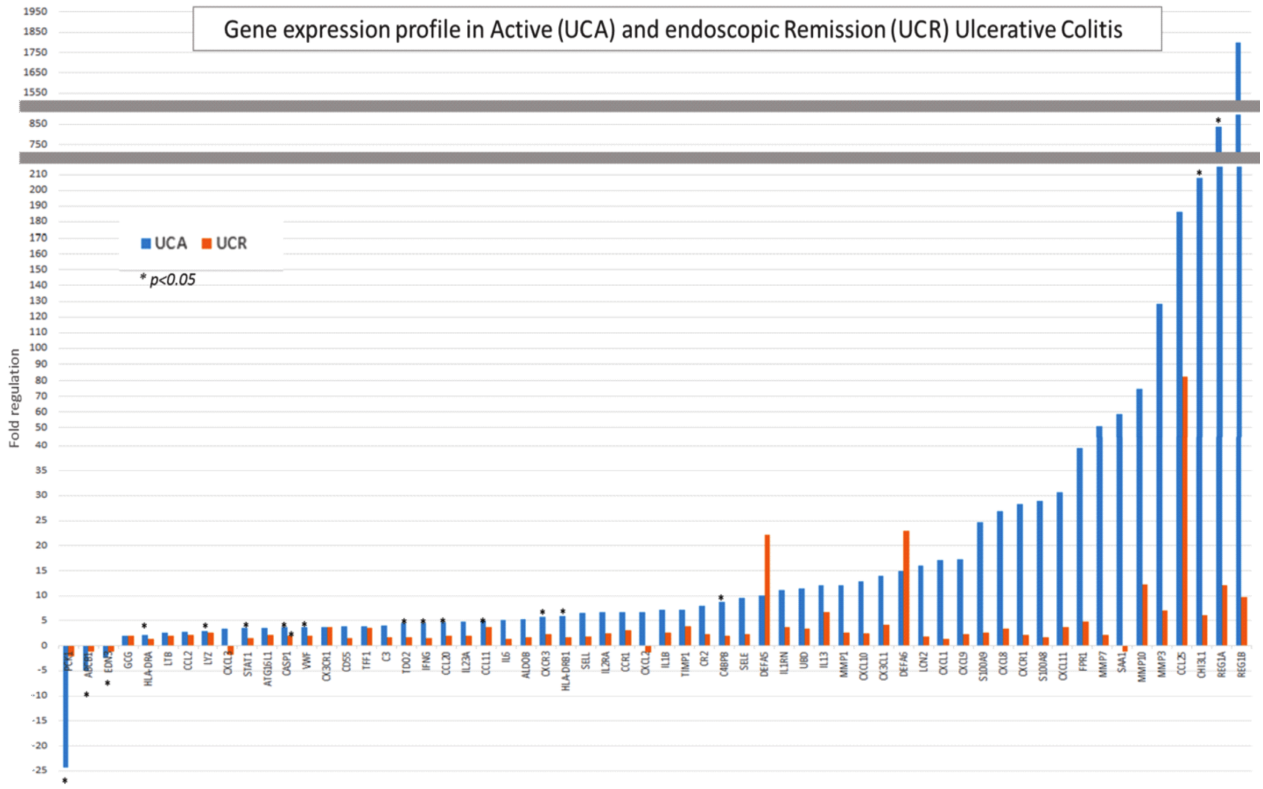

Figure 2 – ***Genes with a fold regulation above 2 in UCA and UCR. UCA: Active ulcerative colitis; UCR: Remission ulcerative colitis.***

Among these, 17 genes were found differentially expressed also in term of statistical significance (p<0.05 and -2>FR>2) in active ulcerative colitis (UCA) comparing pathological *vs.* normal tissue (14 up-regulated and three down-regulated), whereas analysis on ulcerative colitis in remission (UCR) identified in the pathological tissue a single gene down-regulated and two genes up-regulated.

Genes with statistical significance (p<0.05) are marked on the chart with an asterisk (*) and are listed in Table 2.

In Figure 3, patients and genes are clustered based on gene expression similarity. In the colored bar at the bottom of the figure, "green" indicates the lowest and "red" indicates the highest expression.

We found a significant Pearson's correlation between *REG1A*, *CHI3L1* log_2(fold change) levels and severity of the lesions, both evaluated by Mayo (*REG1A* gene, p=0.033, r=0.747; *CHI3L1* gene, p=0.012, r=0.822) and Nancy (*REG1A* gene, p=0.029, r=0.759; *CHI3L1* gene, p=0.007, r=0.851).

No correlation was observed between severity of the lesions and *ISG15* gene levels (p>0.05).

Figure 4 illustrates the box plots (A) and correlations with the severity of histological changes expressed by Nancy score (B) of the most significant genes (*REG1A*, *CHI3L1*, and *ISG15*).

The upper line of the box marks the 75th percentile, the middle line is the median value and the lower line specifies the 25th percentile. Whiskers above and below the box indicate the 90th and 10th percentiles, respectively).

Table 2 – ***Genes differentially expressed between UCA and UCR vs. controls***

Comparison	Pathological tissue *vs.* normal tissue			
Study group	Group A (active disease – UCA)		Group B (remission disease – UCR)	
Gene	*Fold regulation*	*p-value*	*Fold regulation*	*p-value*
ABCB1	-4.9	***0.006***	-1.2	0.959
C4BPB	8.8	***0.028***	2.0	0.364
CASP1	3.7	***0.040***	2.1	***0.040***
CCL11	5.0	***0.015***	3.8	0.159
CCL20	4.6	***0.023***	1.9	0.284
CHI3L1	207.8	***0.038***	6.1	0.157
CXCR3	5.8	***0.006***	2.4	0.188
EDN3	-2.4	***0.035***	-1.1	0.819
HLA-DRA	2.1	***0.002***	1.3	0.355
HLA-DRB1	6.0	***0.002***	1.7	0.464
IFNG	4.6	***0.015***	1.5	0.315
ISG15	1.1	*0.459*	-2.2	***0.036***
LYZ	2.9	***0.010***	2.6	***0.049***
PCK1	-24.3	***<0.001***	-2.2	0.342
REG1A	838.7	***0.040***	12.1	0.263
STAT1	3.6	***0.006***	1.6	0.172
TDO2	4.5	***0.029***	1.6	0.245
VWF	3.8	***0.022***	2.0	0.062

UCA: Active ulcerative colitis; UCR: Remission ulcerative colitis.

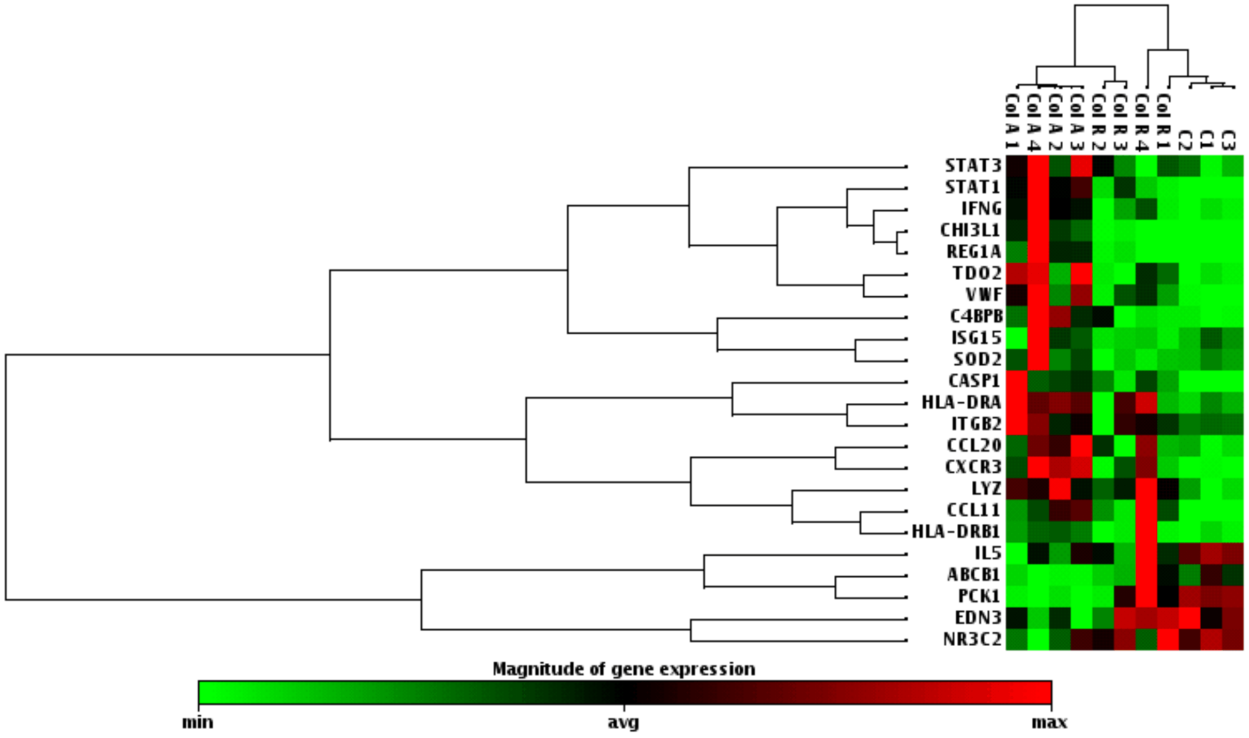

Figure 3 – ***Hierarchical clustering analysis of gene expression in colonic samples from patients with UCA, UCR and controls. UCA: Active ulcerative colitis; UCR: Remission ulcerative colitis.***

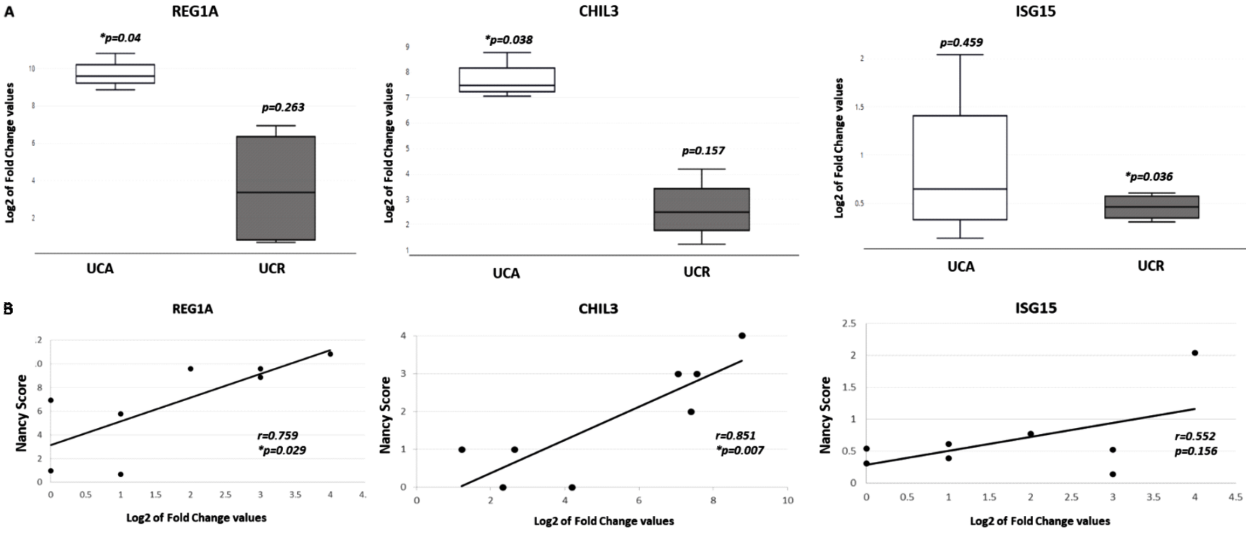

Figure 4 – ***(A) Box plot of the most significant genes (REG1A, CHIL3, ISG15). (B) Correlations between Nancy score and log$_2$ of the fold change of the most significant genes. UCA: Active ulcerative colitis; UCR: Remission ulcerative colitis.***

Discussion

In this study, we investigated for the first time the mucosal gene expression of a large panel of transcripts belonging to pathways associated to IBD mechanisms in a small series of Romanian population affected by ulcerative colitis and controls.

Our results showed that among the significant transcripts, *REG1A* and *CHI3L1* genes reported an up-regulation in UCA, with a fold difference above 200. Moreover, a positive correlation between the severity of the disease and the levels of these genes were observed. In UCR, only *ISG15* gene seemed to be specifically associated to the remission state.

Even though this is a small case-to-case study, some general trending features in mucosal gene expression can be observed. Our results are similar to previous gene expression studies showing patterns of overexpression indicative of chronic inflammation [12, 13].

Regenerating family member genes (*REG*) showed increased expression during IBD disease activity (acute and chronic). Previous studies have shown that *REG1A* and *REG1B* expressed by metaplastic colonic Paneth cells are present both in inflamed and un-inflamed biopsies [14]. In our study, all patients with active disease had *REG1A* and *REG1B* expression highly up-regulated (highest fold regulation, more than 1.500), while for patients in remission a lower increase was observed (only

in two patients, no more than 200 fold regulation), without reaching statistical significance in the case of *REG1B* gene.

Another interesting gene in our study is represented by chitinase 3-like 1 (*CHI3L1*), which interacts with Toll-like receptor 4 (TLR4), permitting the adhesion and the invasion of bacteria in colonic epithelia. Some studies suggested that *CHI3L1* could be useful in detecting early dysplasia in IBD patients, when compared to normal patients [15]. Our results showed a significant up-regulation of *CHI3L1* gene, only in UCA patients. However, in some UCR patients, its levels were still high. This may indicate that, despite the complete remission observed at endoscopy levels, some molecular mechanisms related to the disease are still active.

In UCA cohort, other genes have been found up-regulated with a smaller, but significant fold regulation and, of note, some of them have been previously found involved in UC. One example is the association between human leukocyte antigen (HLA) class II genes, especially HLA-DR and DQ phenotypes in IBD [16]. Multiple studies have reported associations between the presence of *DRB1* alleles in biopsies from colorectal cancer in IBD patients [17].

Interestingly, significant differences in UCA were observed also for lysozyme (*LYZ*) and caspase-1 (*CASP1*) genes. Host defense response to pathogens in the colon is modulated by *LYZ* and cysteine protease *CASP1* that lead to IL-1β and IL-18 synthesis. These genes have been found expressed at different levels in colonic biopsies of IBD and irritable bowel syndrome (IBS) patients [18]. Immunohistochemical studies have previously documented lysozyme up-regulation in metaplastic colonic Paneth cells of both CD and UC even in remission [19].

Signal transducer and activator of transcription 1 (STAT1) protein family is activated after phosphorylation by Janus kinases (JAKs) and can be mainly detected in monocytic cells and neutrophils in inflamed mucosa of UC patients with better correlation to endoscopic score when compared to Mayo score [20]. In our study, up-regulation of *STAT1* gene in UCA patients was variable, without reaching statistical significance.

Among the three down-regulated genes in UCA patients, phosphoenolpyruvate carboxykinase 1 (*PCK1*) showed the largest down-regulation. PCK1 is an enzyme involved in the gluconeogenesis pathway, which, in line with our results, has been reported to show down-regulation in IBD [21]. However, the exact mechanism by which it acts and its implications in clinical practice are not clear.

Regarding genes differentially expressed in UCR, only interferon-stimulated gene 15 (*ISG15*) was specifically down-regulated. *ISG15* codify for *ISG15* ubiquitin-like modifier protein and it is involved in interferon-induced defense against pathogen invasion. Of note, it is regulated by microtubule-associated serine/threonine kinase 3 (*MAST3*), an IBD susceptibility gene that modulates the inflammatory response [22].

Recent technological advances allowed for complex quantification of inflammatory expression patterns in normal and inflamed tissue. Identifying dysregulated genes may lead to future therapeutic targets, early biomarkers of disease and might help differentiate between the two entities (UC and CD), if clinical and endoscopic aspects overlap. Furthermore, identifying over-regulated genes in endoscopic normal mucosa could predict relapses or define profound remission in UC patients.

Conclusions

In this study, we identified many genes significantly associated with UCA and UCR, out of which *ISG15* particularly seemed to be specifically associated to the UC remission state. However, a limitation should be discussed: the study is based on a small cohort of eight patients and three controls and findings should be reproduced in a larger cohort.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

The authors thank the patients involved in the study for the generous collaboration. This research was funded by project PN 16.22.03.01 with support of the National Authority for Scientific Research and Innovation (ANCSI through "Nucleu" Program).

Author contribution

The first and the second authors had equal contribution to the achievement of the article.

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Received: March 30, 2017

Accepted: February 14, 2018