ACTA ABP BIOCHIMICA POLONICA

Vol. 62, No 1/2015
69–75
http://dx.doi.org/10.18388/abp.2014_778

Regular paper

Transcriptional changes between uninflamed ulcerative colitis and familial adenomatous polyposis pouch mucosa can be attributed to an altered immune response

Agnieszka Paziewska[1#], Karolina Horbacka[2#], Krzysztof Goryca[3], Michal Mikula[3], Dorota Jarosz[1,3], Michalina Dabrowska[3], Piotr Krokowicz[2], Jacek Karon[2] and Jerzy Ostrowski[1,3]✉

[1]Department of Gastroenterology and Hepatology, Medical Center for Postgraduate Education, Warsaw, Poland; [2]Department of General and Colorectal Surgery, Poznan University of Medical Sciences, Poznań, Poland; [3]Department of Genetics, Maria Sklodowska-Curie Memorial Cancer Center and Institute of Oncology, Warsaw, Poland

A total proctocolectomy with ileal pouch-anal anastomosis (IPAA) is considered the surgery of choice for definitive management of familial adenomatous polyposis (FAP) and some patients with ulcerative colitis (UC). However, this surgical treatment is often associated with pouchitis, a long-term complication that occurs mostly in UC patients. The purpose of this study was to better define the molecular background of pouchitis. A microarray-based survey was performed using pouch mucosal samples collected from 28 and 8 patients undergoing surgery for UC and FAP, respectively. There were 4,770 genes that significantly differentiated uninflamed from inflamed mucosal samples, and their functional features were represented mostly by metabolic and cell proliferation pathways. In contrast, functional analyses of aberrantly expressed genes between UC and FAP samples, irrespective of mucosal inflammation status, revealed multiple pathways and terms that were linked to changes in immune response. Interestingly, the comparison of uninflamed UC and FAP samples identified a set of 29 altered probe sets, including an inflammation-related transcript encoding a Charcot-Leyden crystal (CLC) protein. The most distinct changes in gene expression profiles differentiating uninflamed UC and FAP pouch mucosal samples were attributed to the Gene Ontology category *innate immune response*. Our study confirmed that alterations in immune responses can be found between patients who underwent surgery for UC and FAP, independent of the pouch inflammation status. This observation may be important when managing IPAA patients.

Key words: ulcerative colitis; familial adenomatous polyposis; pouch, gene expression; immune response

Received: 22 April, 2014; revised: 04 November, 2014; accepted: 12 November, 2014; available on-line: 04 February, 2015

INTRODUCTION

All patients with familial adenomatous polyposis (FAP) and up to 30% of ulcerative colitis (UC) patients with refractory disease or colitis-associated dysplasia require a total colectomy (Mahadevan & Sandborn, 2003; Cosnes *et al.*, 2011; Li & Shen, 2012). Although the restorative proctocolectomy with ileal pouch-anal anastomosis (IPAA) decreases the risk of carcinogenesis and improves quality of life by preserving intestinal continuity, side effects occur frequently (Li & Shen, 2012). Pouchitis is the most common long-term complication of IPAA and develops after exposure of the pouch mucosa to fecal stasis (Mahadevan & Sandborn, 2003; Gionchetti *et al.*, 2004; Ohge *et al.*, 2005; Yamamoto *et al.*, 2005; Li & Shen, 2012). The etiology of pouchitis is considered multifactorial.

FAP and UC are two disorders with very different pathogeneses. FAP is a hereditary syndrome, which predisposes towards multiple colonic adenomas that exhibit a 100% risk of malignancy (Stec *et al.*, 2010). UC is an immune-mediated disorder of the large intestine that develops in genetically predisposed individuals (Baumgart & Carding, 2007). Since pouchitis affects up to 50% of patients with UC and only 5% of patients with FAP (Leal *et al.*, 2010a), it is commonly speculated that pouchitis occurring in UC patients may reflect a recurrence of the UC-related inflammatory response to the ileal mucosa.

UC results from abnormal activation of both innate and adaptive immune responses to intestinal flora (Bouguen *et al.*, 2011). When the balance between pro- and anti-inflammatory cytokines and the suppressors of cytokine signaling proteins is impaired, it can mediate interactions between activated immune cells and epithelial and mesenchymal cells that lead to an abnormal inflammatory response (Bouguen *et al.*, 2011). The development of pouchitis is also associated with higher levels of TNF-α, IL-1β, IL-6, and IL-8 and decreased levels of IL-10 (reviewed in Leal *et al.*, 2010b) in UC patients, but not FAP patients. Moreover, even in normal pouch mucosa, significant differences were found in the levels of inflammation activity markers between UC and FAP patients (Leal *et al.*, 2010b; de Paiva *et al.*, 2011). Thus, although the molecular mechanisms underlying both UC and UC-related pouchitis are only partly defined, it is reasonable that pouchitis is a recurrence of UC in the pouch mucosa.

✉e-mail: jostrow@warman.com.pl
#These authors contributed equally to this work

Abbreviations: IPAA, ileal pouch-anal anastomosis; FAP, familial adenomatous polyposis; UC, ulcerative colitis; PDAI, the pouchitis disease activity index; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; qRT-PCR; quantitative Reverse-Transcriptase-PCR; FDR, false discovery rate; FC, fold change; K-S, Kolmogorov-Smirnov

Microarray-based studies can identify genes that are differentially expressed between predefined groups of samples; gene subsets with differential expression across predefined classes of genes; and gene expression profiles that determine gene expression signatures among seemingly homogenous phenotypes (Ostrowski & Wyrwicz, 2009). Gene expression profiling does not require prior knowledge of the expression profile or a pre-existing hypothesis about the differential gene expression typical for a given biological phenomenon. The results of large-scale measurements can be further analyzed by term enrichment approaches, which may indicate the altered signaling pathways in large-scale cellular networks.

Recently published studies, evaluating molecular profiles in pouch mucosa, have reported that genes significantly associated with UC outcome are involved in regulation of the immune system, modification of the extracellular matrix, and xenobiotic activity (Kabakchiev *et al.*, 2014), and gene alterations in pouch inflammation overlap with alterations within proximal small bowel (Yanai *et al.*, 2014) and Crohn's disease (Ben-Shachar *et al.*, 2013).

In this study, a microarray-based survey was performed to better define the molecular background of UC-related pouchitis and to identify differences in the pouch mucosal defense pathways between UC and FAP.

MATERIALS AND METHODS

Patients. Thirty six patients (27 men) with IPAA were enrolled in the study. The patients were recruited at the Outpatient Clinic of the Department of General and Colorectal Surgery during the periodic health evaluation. Among them, 28 patients (median age, 41; range, 27–66 years) underwent surgery for UC and 8 patients (median age, 25; range 19–51 years) for FAP. The median follow-up after the surgery was 24 months; range, 9–142 months, and all the patients had had their ileostomy closed at the time of the study. The Pouchitis Disease Activity Index (PDAI) was used to diagnose or exclude pouchitis (Sandborn *et al.*, 1994; Shen *et al.*, 2002). For each patient, six biopsies were taken from the same areas of the lower part of the pouch mucosa (above the rectal cuff) during endoscopic examination. The reservoir design was the 'J' type. Three biopsy specimens taken for molecular testing were immediately snap-frozen and stored at –72°C until use. The other three biopsy specimens were fixed in 10% formalin for histological examination, and paraffin sections (4 μm thick) were routinely stained with hematoxylin and eosin.

The study was performed in accordance with the Declaration of Helsinki. The study protocol was approved by the Medical Sciences Bioethics Committee at Poznan University and all patients signed informed consent before inclusion.

mRNA extraction. Total RNA was isolated from mucosal biopsies using the RNeasy Plus Mini Kit (Qiagen, Germany) followed by on-column DNase I digestion. The quality of RNA samples was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA); all samples used for microarray analysis displayed distinct peaks corresponding to intact 28S and 18S ribosomal RNA.

Quantitative(q) RT-PCR measurements. QRT-PCR was performed as described before (Mikula *et al.*, 2011) using Sybr Green chemistry. Geometric mean expression of Ubiquitin C — UBC and ribosomal protein P0 — RPLP0 was used as a normalization factor as a result of evaluation of three housekeeping genes (ACTB - actin beta, UBC and RPLP0) with the GeNorm software (Vandesompele *et al.*, 2002). Gene expression results were calculated using ΔΔ Ct method (Livak & Schmittgen, 2001). Primers for the analyzed gene transcripts were taken from PrimerBank (Wang *et al.*, 2011). The results were analyzed with Mann-Whitney U test using GraphPad Prism 5 software (GraphPad Software, Inc.). *P* values of less than 0.05 were considered as significant.

Gene expression microarray analysis. The average bead signals from the Human HT-12 v4 Expression BeadChip kit (AROS Applied Biotechnology, Aarhus N, Denmark) were quantile normalized with no background correction. All computations were done using R 2.15.0 software with the Bioconductor extension (Gentleman *et al.*, 2004). Measurements were filtered according to the relation of interquartile range (IQR) to median. Only probes with IQR/median >1/8 were selected for analysis and 24 050 probes out of 47 231 passed filtering. Genes with differential expression were selected according to the *p*-value from the *t*-test (Welch variant) after correction for multiple hypothesis testing with the Benjamini-Hochberg algorithm. Adjusted *p*-values <0.05 were considered significant. Hierarchical clustering was performed using a complete method with Euclidean distance as the dissimilarity measure.

Illumina identifiers were mapped to genes and Kyoto Encyclopedia of Genes and Genomes (KEGG) identifiers using the lumiHumanAll.db (1.16.0), KEGG.db (2.6.1), and lumi (2.6.0) (Du *et al.*, 2008) packages. The significance of expression changes related to each KEGG pathway was assessed with the Kolmogorov-Smirnov (K-S) test using probe lists sorted by the appropriate *p*-value as input. Mapping to Gene Ontology (GO) (Ashburner *et al.*, 2000) terms was performed with the GO.db (2.6.1) package (Lin *et al.*, 2008). The significance of expression changes related to the GO terms (biological function branch) was assessed with the hypergeometric test. Results from the K-S and hypergeometric tests were corrected for multiple hypothesis testing with the Benjamini-Hochberg algorithm.

Microarray data were deposited in Gene Expression Omnibus under GSE50788.

RESULTS

Signaling pathways that differed between uninflamed and inflamed pouch mucosa

At the time of the study, uninflamed pouch mucosa was found in 15 and 5 patients with IPAA who underwent surgery for UC and FAP, respectively, by both endoscopic and histopathologic evaluation (the PDAI histology score = 0), and pouchitis was diagnosed in 13 UC and 3 FAP patients. In the latter patients mild to moderately active pouchitis was diagnosed clinically (increased stool frequency, fecal urgency or abdominal cramp, fever, rectal bleeding), endoscopically (≥4 findings of granularity, edema, friability, loss of vascular pattern, mucosal hemorrhage, or ulceration), and histologically (varying degrees of changes in mucosal architecture, infiltration by acute and chronic inflammatory cells, ulceration, and crypt abscesses; mean PDAI histology score was 2.62 ± 1.31) (Shen *et al.*, 2002). On endoscopy, the neoterminal ileum above the pouch was normal in all patients.

To compare gene expression profiles corresponding to tissue morphology, microarray data sets were analyzed using pair-wise comparisons between 20 uninflamed and

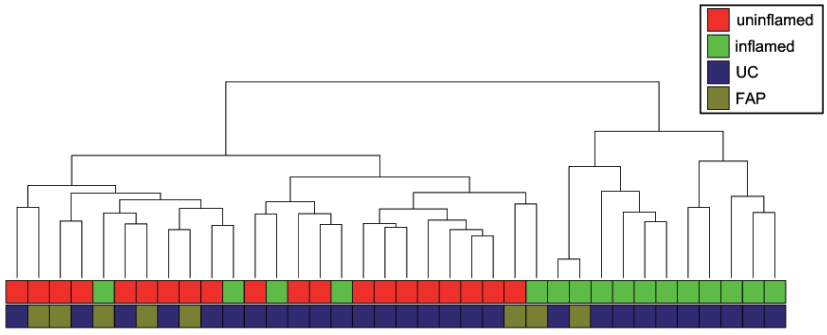

Figure 1. Hierarchical clustering of 36 samples based on their gene expression.
Hierarchical cluster analysis of the whole dataset shows that inflamed samples cluster together. FAP, familial adenomatous polyposis; UC, ulcerative colitis.

16 inflamed pouch mucosal samples from both UC and FAP patients. A total of 24 050 probe sets passed our filtration criterion (IQR/median >1/8). Of these, 7 152 probes (4 770 genes) significantly differentiated (p<0.05, FDR adjusted) uninflamed from inflamed samples and 584 (437 genes) had expression fold changes (FC) greater than 2 (Table S1 at www.actabp.pl). The unsupervised hierarchical clustering grouped mucosa samples according to their inflammation status; although, the distinction between two branches was not absolutely concordant with histological evaluation. As shown in Fig. 1, the inflamed mucosa branch represented 10 out of 13 UC samples and one out of three FAP samples.

Differentially regulated transcripts in comparison of uniflamed and inflamed samples were further evaluated using qRT-PCR. Eight most discriminative genes were chosen for evaluation: four upregulated in inflamed tissue (carcinoembryonic antigen-related cell adhesion molecule 7 — CEACAM7; V-set and immunoglobulin domain containing 2 — VSIG2; carbonic anhydrase 2 — CA2; membrane-spanning 4-domains, subfamily A, member 12 — MS4A12) and four downregulated (cubilin — CUBN; sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone (DHEA)-preferring, member 1 — SULT2A1; flavin containing monooxygenase 1 — FMO1; pancreatic lipase-related protein 2 — PNLIPRP2) (Table S1 at www.actabp.pl). QPCR measurements confirmed differences in mRNA abundances indicated on microarrays for all selected genes (Fig. 2).

The KEGG signaling pathways derived from all gene expression measurements that passed the filtration criterion identified 27 pathways that discriminated uninflamed from inflamed pouch mucosa (Table S2 at www.actabp.pl). While most of the pathways were associated with metabolic processes, the notable exceptions included cell cycle, DNA replication, and RNA transport pathways and the B cell receptor signaling pathway, the single KEGG pathway related to immunological processes. Genes involved in metabolism pathways were usually down-regulated in inflamed specimens, while genes from DNA replication and RNA transport were up-regulated. The probe sets that were differentially expressed between uninflamed and inflamed mucosa samples (with corrected p-values <0.05) were then used for the calculation of their attribution to GO terms. GO analyses uncovered 38 significant terms (Table S3 at www.actabp.pl) that confirmed the findings based on KEGG.

Signaling pathways that distinguish between UC and FAP-related pouch mucosa

Next, similar comparative analyses were performed on microarray data of the mucosal samples, representing the two groups of disorders, UC and FAP, independent of inflammation status. This pair-wise comparison re-

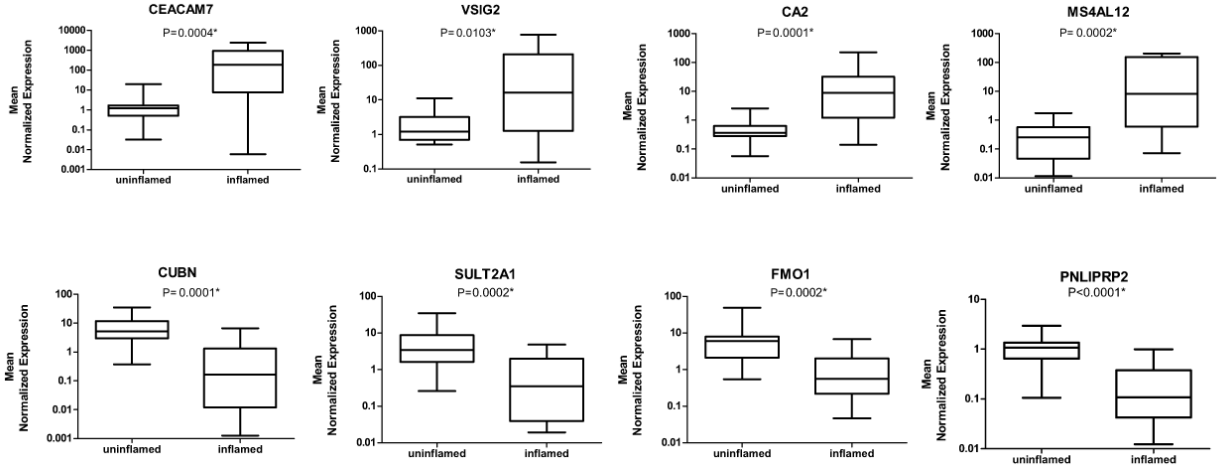

Figure 2. QRT-PCR validation of genes with highest magnitude of changes between uninflamed and inflamed samples in microarray survey.
Relative levels for carcinoembryonic antigen-related cell adhesion molecule 7 — CEACAM7; V-set and immunoglobulin domain containing 2 — VSIG2; carbonic anhydrase 2 — CA2; membrane-spanning 4-domains, subfamily A, member 12 — MS4A12; cubilin — CUBN; sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone (DHEA)-preferring, member 1 — SULT2A1; flavin containing monooxygenase 1 — FMO1 and pancreatic lipase-related protein 2 — PNLIPRP2 were measured in RNA extracted from uninflamed (n=20) and inflamed (n=16) biopsies. Geometric mean expression of UBC and RPLP0 was used for normalization between samples. The box border represents the interquartile range, and the horizontal line in the box is the median. The whiskers denote the largest/smallest observation. The logarithmic scale was used to accommodate the magnitude of expression changes. Statistical significances of differences were assessed by the Mann–Whitney test and P values <0.05 were considered as significant (*).

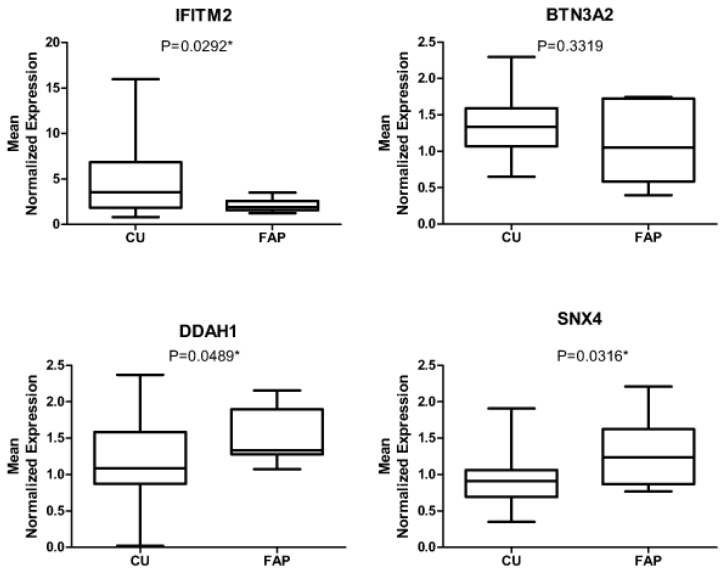

Figure 3. QRT-PCR validation of genes differentiating UC and FAP samples in microarray survey. Relative levels for interferon induced transmembrane protein 2 — IFITM2, butyrophilin, subfamily 3, member A2 — BTN3A2, dimethylarginine dimethylaminohydrolase 1 — DDAH1 and sorting nexin 4 — SNX4 were measured in RNA extracted from UC (n=28) and FAP (n=8) biopsies. Gene expression calculation was performed as in Figure 2. The box border represents the interquartile range, and the horizontal line in the box is the median. The whiskers denote the largest/smallest observation. Statistical significances of differences were assessed by the Mann–Whitney test and *P* values <0.05 were considered as significant (*).

Table 1. List of genes differentially expressed (adjusted *p*-value ≤0.05) between uninflamed UC and FAP samples. In bold are the genes expressed with a fold change >1.5.

Gene name	Symbol	Adj. p-value	Fold change
lysosomal-associated membrane protein 2	LAMP2	0.032	0.83
BRCA2 and CDKN1A interacting protein	BCCIP	0.032	0.80
Charcot-Leyden crystal protein	**CLC**	**0.032**	**1.54**
VAMP (vesicle-associated membrane protein)-associated protein A, 33kDa	VAPA	0.032	0.80
dual specificity phosphatase 2	DUSP2	0.032	1.30
metallo-beta-lactamase domain containing 2	MBLAC2	0.032	0.86
transcriptional adaptor 3	TADA3	0.032	1.33
ubiquitin-conjugating enzyme E2 variant 1	UBE2V1	0.032	1.36
heat shock protein 90kDa alpha (cytosolic), class A member 1	HSP90AA1	0.032	0.78
family with sequence similarity 110, member A	FAM110A	0.039	1.41
inositol 1,4,5-trisphosphate receptor interacting protein-like 2	**ITPRIPL2**	**0.039**	**1.97**
p21 protein (Cdc42/Rac)-activated kinase 7	PAK7	0.039	1.39
acidic (leucine-rich) nuclear phosphoprotein 32 family, member C	ANP32C	0.039	0.85
inhibitor of growth family, member 3	ING3	0.039	0.77
proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional peptidase 7)	PSMB8	0.039	1.35
nuclear transcription factor, X-box binding 1	NFX1	0.039	1.29
microtubule associated serine/threonine kinase 3	MAST3	0.043	1.36
ring finger protein 38	RNF38	0.045	0.82
component of oligomeric golgi complex 5	**COG5**	**0.045**	**1.72**
ubiquitin protein ligase E3C	UBE3C	0.045	0.85
sperm antigen with calponin homology and coiled-coil domains 1	SPECC1	0.045	1.40
leucine rich repeat containing 58	LRRC58	0.046	0.68
NECAP endocytosis associated 2	NECAP2	0.047	1.27
dimethylarginine dimethylaminohydrolase 1	DDAH1	0.047	0.72
Der1-like domain family, member 3	DERL3	0.047	1.22

vealed only 48 probe sets (40 genes) as significantly different ($p<0.05$, FDR adjusted) between the two groups of samples (Table S4 at www.actabp.pl). To validate the expression of the most altered genes in UC and FAP comparison the qRT-PCR was performed to ascertain their discriminative status. We selected two upregulated

Table 2. Gene ontology (GO) terms corresponding to the top five percent probe sets differentiating between uninflamed FAP and UC mucosa samples.
Probe sets were sorted according to their p-value as an input for GO analyses. Enrichment of GO terms (adj. p-value ≤0.01) was assessed in a background of all probe sets that passed filtration. The GO with p-value <0.001 were included to underline overrepresentation of immune-related terms in probe sets annotation.

GO ID	Terms	p-value	Adj. p-value
GO:0045087	innate immune response	1.93E-06	7.79E-03
GO:0051704	multi-organism process	1.15E-05	2.31E-02
GO:0044248	cellular catabolic process	0.000134	1.61E-01
GO:0006954	inflammatory response	0.000159	1.61E-01
GO:0070586	cell-cell adhesion involved in gastrulation	0.000236	1.91E-01
GO:0032535	regulation of cellular component size	0.000361	1.99E-01
GO:0002250	adaptive immune response	0.000401	1.99E-01
GO:0006953	acute-phase response	0.000407	1.99E-01
GO:0002682	regulation of immune system process	0.000476	1.99E-01
GO:0009057	macromolecule catabolic process	0.000526	1.99E-01
GO:0030307	positive regulation of cell growth	0.000543	1.99E-01
GO:0031334	positive regulation of protein complex assembly	0.000636	2.14E-01
GO:0045732	positive regulation of protein catabolic process	0.000742	2.14E-01
GO:0080134	regulation of response to stress	0.000764	2.14E-01
GO:0006950	response to stress	0.000812	2.14E-01
GO:0019915	lipid storage	0.000892	2.14E-01
GO:0032825	positive regulation of natural killer cell differentiation	0.000902	2.14E-01

(interferon induced transmembrane protein 2 — IFITM2 and butyrophilin, subfamily 3, member A2 — BTN3A2) and two downregulated (dimethylarginine dimethylaminohydrolase 1 — DDAH1 and sorting nexin 4 — SNX4) genes (Table S4 at www.actabp.pl). QPCR survey confirmed significantly changed mRNA abundance of IFITM2, DDAH1 and SNX4 gene (Fig. 3).

While the K-S test used for the annotation of gene expression measurements to pre-defined KEGG signaling pathways showed their attribution to 22 pathways (Table S5 at www.actabp.pl), a small number of differentiating genes below the applied significance threshold made it impossible to select GO terms using the hypergeometric test. The disparity between KEGG and GO functional analyses stems from different statistical approaches; the latter one requires a set of differentiating genes fulfilling certain statistical conditions.

To further explore if GO terms could portray UC *vs.* FAP functional changes in pouch mucosa, similarly to KEGG, we used the over-representation method for testing the top five percent of probe sets, sorted according to their *p*-value, as the input for GO analyses. This approach identified 31 GO terms that were in close agreement with the KEGG findings. The annotation results showed mostly immunologically related KEGG pathways, including those associated with B-cell, T-cell, and toll-like (TLR) receptors (Table S5 at www.actabp.pl) and GO categories associated with immune response (Table S6 at www.actabp.pl).

Signaling pathways that distinguish normal pouch mucosa between UC and FAP patients

Pair wise comparison of uninflamed UC and FAP samples identified that there was altered expression of 29 probe sets ($p<0.05$, FDR adjusted) belonging to 25 genes (Table 1). The expression status of three selected genes (inositol 1,4,5-trisphosphate receptor interacting

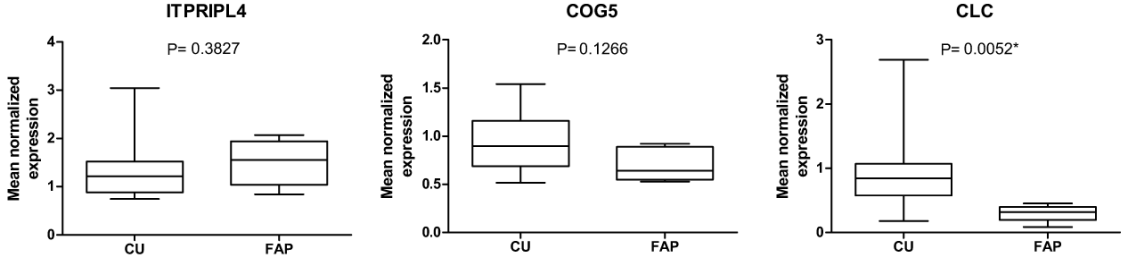

Figure 4. QRT-PCR validation of genes differentiating uninflamed UC and FAP samples in microarray survey.
Relative levels for inositol 1,4,5-trisphosphate receptor interacting protein-like 2 (ITPRIPL2), component of oligomeric golgi complex 5 (COG5) and Charcot-Leyden crystal protein (CLC) were measured in RNA extracted from uninflamed UC (n=15) and FAP (n=5) biopsies. Gene expression calculation was performed as in Fig. 2. The box border represents the interquartile range, and the horizontal line in the box is the median. The whiskers denote the largest/smallest observation. Statistical significances of differences were assessed by the Mann–Whitney test and *P* values <0.05 were considered as significant (*).

protein-like 2 — ITPRIPL2; component of oligomeric golgi complex 5 — COG5 and Charcot-Leyden crystal protein — CLC), that demonstrated expression greater than 1.5-fold, was independently validated by qRT-PCR. While the direction of expression changes of two genes, COG5 and CLC, was consistent with microarray measurement, only the expression of CLC mRNA was significantly changed by 3.1-fold between uninflamed UC and FAP samples (Fig. 4).

Under standard testing conditions, no functional processes or terms could be selected using KEGG or GO analyses, respectively. However, when using the top five percent of probe sets (according to their *p*-value) differentiating uninflamed mucosa from CU and uninflamed mucosa from FAP patients, the GO analysis indicated an inflammation related term: innate immune response (GO:0045087) (Table 2).

DISCUSSION

The frequency of pouchitis is much lower in patients surgically treated for FAP than for UC, suggesting that inherent molecular properties of ileal mucosa have a critical role in its pathogenesis. If so, determination of the molecular makeup of FAP and UC pouches may provide clues to understanding the mechanisms underlying the development of pouchitis. The frequencies of pouchitis in FAP and UC suggested there are pathophysiological similarities related to immune response in UC and pouchitis after surgery. This hypothesis was previously tested in a few published studies (Leal *et al*., 2010*b*; de Paiva *et al*., 2011) that aimed to connect molecular changes within the UC pouch with inflammation. It was determined that the signal transducer IFN-γ and an activator of cytokine expression STAT1 were increased in the normal pouch mucosa of UC patients (Leal *et al*., 2010*b*). Furthermore, UC exhibited significantly higher levels of TLR4 protein than FAP patients; TLR4 is a receptor involved in the recognition of extracellular cues from bacteria and activation of the innate immune system (de Paiva *et al*., 2011). Although these studies were performed on singular elements involved in immune response, the results led to the same conclusion that inflammatory-like properties of the UC pouch could explain the more frequent onset of pouchitis.

Most disorders result from the dysfunction of signaling pathways in large-scale cellular networks. Therefore, analyses of disrupted cellular components and the related aberrant signaling may be the key factors in studies on the molecular basis of diseases. A comparison of the gene expression profiles corresponding to the inflamed mucosal morphology showed numerous genes with altered expression, which met the FDR threshold criteria for being considered differentially expressed (Table S1 at www.actabp.pl). Probe sets sorted according to significant differences between uninflamed and inflamed pouch mucosal samples uncovered several GO categories related to very similar metabolic dysfunctions based on KEGG annotations (Table S2 and S3 at www.actabp.pl). Surprisingly, the vast majority of the altered pathways were not related to immune response. Further KEGG and GO analyses of probe sets with expression differences between UC and FAP samples, irrespective of inflammation status, revealed multiple pathways (Table S5 at www.actabp.pl) and terms (Table S6 at www.actabp.pl), respectively, linked to immune response. When comparing uninflamed UC and FAP samples, we identified 25 genes with differential expression (Table 1). However, only 3 of these genes demonstrated expression greater than 1.5-fold, indicating that the established set of differentially expressed transcripts reflect modest changes. Further validation with qRT-PCR confirmed altered expression of mRNA encoding CLC gene (Fig. 4). The CLC protein has lysophospholipase activity and is expressed in eosinophils and basophils. Its level has been shown to correlate with eosinophil density and the degree of inflammation in celiac disease (De Re *et al*., 2009). Furthermore, its mRNA abundance was 10-times higher in early- *versus* late-onset colorectal tumors (Ågesen *et al*., 2011), where early tumors displayed higher overall expression of immune-relevant genes. Of note, the most distinct changes in gene expression profiles differentiating uninflamed UC and FAP mucosa samples could be attributed to the GO category *innate immune response* (Table 2), but not the KEGG pathway, when using the top five percent of differentiating probe sets. Therefore, by using the whole transcriptome survey, for the first time, we provide indirect evidence that molecular mechanisms underlying inflammation of ileal pouch mucosa in UC patients can mimic those associated with the development of immune-mediated inflammation in the large intestine. Interestingly, previous work comparing the whole transcriptomes of biopsies obtained from UC patients with and without active large bowel inflammation also provided evidence for the molecular signature of the "pre-inflammatory state" in histologically uninflamed UC colonic specimens (Olsen *et al*., 2009).

While the microarray technology allows surveying changes in gene expression in genome scale, the results are often required to be verified using qPCR, specifically when potential biomarkers of disease need to be validated. QPCR technique is considered as a gold standard in gene expression measurements, however the concordance of qPCR and microarray data may be significantly influenced by the variability of technical procedures inherent to each method. This problem has already been explored and discussed in the literature (Morey *et al*., 2006). Among the other factors, a small degree of gene expression change, typically of less than 2-fold, has been determined as the feature lowering correlations between microarrays and qPCR (Etienne *et al*., 2004; Morey *et al*., 2006). This could be reflected by the reproducibility of microarrays and qRT-PCR results for a set of altered genes in two comparisons performed on pouch mucosas: uninflamed *vs*. inflamed (Fig. 2) and CU *vs*. FAP (Fig. 3), where the magnitude of expression changes and the number of differentiating genes for each set was substantially different. We were able to confirm the expression concordance of all genes selected for uninflamed vs. inflamed samples comparison (Fig. 2). At the same time for UC and FAP comparison, where the most discriminative gene (IFITM2) had the expression FC of 2, we confirmed altered expression of three out of four genes tested (Fig. 3). Consequently, poor reproduction of microarrays by qRT-PCR observed for comparison of normal pouch mucosa between UC and FAP patients could be attributed to both the modest changes in expression of the assessed genes (Table 1 and Fig. 4) and the different methodology behind these two techniques.

In summary, the full discovery and understanding of biological processes underlying UC-related pouchitis on a genomic scale would require an extensive knowledge of signaling mechanisms underlying the development of inflammation in UC patients. This knowledge is currently not available. However, our analyses indicate there are significant molecular differences between patients undergoing surgery for UC and FAP, independent of the

pouch inflammation status. This observation may be important when managing IPAA patients.

Acknowledgements

This work was supported by 2011/02/A/NZ5/00339 grant from the National Science Centre and CMKP grant 5011091212.

REFERENCES

Ågesen TH, Berg M, Clancy T, Thiis-Evensen E, Cekaite L, Lind GE, Nesland JM, Bakka A, Mala T, Hauss HJ, Fetveit T, Vatn MH, Hovig E, Nesbakken A, Lothe RA, Skotheim RI (2011) CLC and IFNAR1 are differentially expressed and a global immunity score is distinct between early- and late-onset colorectal cancer. *Genes and Immunity* **12**: 653–662.

Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G (2000) Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet* **25**: 25–29.

Baumgart DC, Carding SR (2007) Inflammatory bowel disease: cause and immunobiology. *Lancet* **369**: 1627–1640.

Bouguen G, Chevaux J-B, Peyrin-Biroulet L (2011) Recent advances in cytokines: therapeutic implications for inflammatory bowel diseases. *World J Gastroenterol* **17**: 547–556.

Cosnes J, Gower-Rousseau C, Seksik P, Cortot A (2011) Epidemiology and natural history of inflammatory bowel diseases. *Gastroenterology* **140**: 1785–1794.

Du P, Kibbe WA, Lin SM (2008) lumi: a pipeline for processing Illumina microarray. *Bioinformatics* **24**: 1547–1548.

Etienne W, Meyer MH, Peppers J, Meyer RA Jr (2004) Comparison of mRNA gene expression by RT-PCR and DNA microarray. *BioTechniques* **36**: 618–620, 622, 624–626.

Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JYH, Zhang J (2004) Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* **5**: R80.

Gionchetti P, Morselli C, Rizzello F, Romagnoli R, Campieri M, Poggioli G, Laureti S, Ugolini F, Pierangeli F (2004) Management of pouch dysfunction or pouchitis with an ileoanal pouch. *Best Pract Res Clin Gastroenterol* **18**: 993–1006.

Kabakchiev B, Tyler A, Stempak JM, Milgrom R, Silverberg MS (2014) Downregulation of expression of xenobiotic efflux genes is associated with pelvic pouch inflammation in ulcerative colitis. *Inflamm Bowel Dis* **20**: 1157–1164.

Leal RF, Ayrizono M de LS, Milanski M, Fagundes JJ, Moraes JC, Meirelles LR, Velloso LA, Coy CSR (2010a) Detection of epithelial apoptosis in pelvic ileal pouches for ulcerative colitis and familial adenomatous polyposis. *J Transl Med* **8**: 11.

Leal RF, Ayrizono MLS, Milanski M, Coope A, Fagundes JJ, Velloso LA, Coy CSR (2010b) Activation of signal transducer and activator of transcription-1 (STAT-1) and differential expression of interferon-gamma and anti-inflammatory proteins in pelvic ileal pouches for ulcerative colitis and familial adenomatous polyposis. *Clin Exp Immunol* **160**: 380–385.

Lin SM, Du P, Huber W, Kibbe WA (2008) Model-based variance-stabilizing transformation for Illumina microarray data. *Nucleic Acids Res* **36**: e11.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**: 402–408.

Li Y, Shen B (2012) Evaluating pouch problems. *Gastroenterol Clin North Am* **41**: 355–378.

Mahadevan U, Sandborn WJ (2003) Diagnosis and management of pouchitis. *Gastroenterology* **124**: 1636–1650.

Mikula M, Rubel T, Karczmarski J, Goryca K, Dadlez M, Ostrowski J (2011) Integrating proteomic and transcriptomic high-throughput surveys for search of new biomarkers of colon tumors. *Functional, Integrative Genomics* **11**: 215–224.

Morey JS, Ryan JC, Van Dolah FM (2006) Microarray validation: factors influencing correlation between oligonucleotide microarrays and real-time PCR. *Biol Proced Online* **8**: 175–193.

Ohge H, Furne JK, Springfield J, Rothenberger DA, Madoff RD, Levitt MD (2005) Association between fecal hydrogen sulfide production and pouchitis. *Dis Colon Rectum* **48**: 469–475.

Olsen J, Gerds TA, Seidelin JB, Csillag C, Bjerrum JT, Troelsen JT, Nielsen OH (2009) Diagnosis of ulcerative colitis before onset of inflammation by multivariate modeling of genome-wide gene expression data. *Inflamm Bowel Dis* **15**: 1032–1038.

Ostrowski J, Wyrwicz LS (2009) Integrating genomics, proteomics and bioinformatics in translational studies of molecular medicine. *Expert Rev Mol Diagn* **9**: 623–630.

De Paiva NM, Ayrizono MLS, Milanski M, Coope A, Oliveira LMF, Fagundes JJ, Velloso LA, Coy CSR, Leal RF (2011) Differential expression of TLR2, TLR4 and JNK in mucosa of ileal pouches for ulcerative colitis. Is there a role for bacterial antigen pathway in asymptomatic patients? *Int J Clin Exp Med* **4**: 179–186.

De Re V, Simula MP, Cannizzaro R, Pavan A, De Zorzi MA, Toffoli G, Canzonieri V (2009) Galectin-10, eosinophils, and celiac disease. *Ann N Y Acad Sci* **1173**: 357–364.

Sandborn WJ, Tremaine WJ, Batts KP, Pemberton JH, Phillips SF (1994) Pouchitis after ileal pouch-anal anastomosis: a Pouchitis Disease Activity Index. *Mayo Clin Proc* **69**: 409–415.

Ben-Shachar S, Yanai H, Baram L, Elad H, Meirovithz E, Ofer A, Brazowski E, Tulchinsky H, Pasmanik-Chor M, Dotan I (2013) Gene expression profiles of ileal inflammatory bowel disease correlate with disease phenotype and advance understanding of its immunopathogenesis. *Inflamm Bowel Dis* **19**: 2509–2521.

Shen B, Achkar J-P, Lashner BA, Ormsby AH, Brzezinski A, Soffer EE, Remzi FH, Bevins CL, Fazio VW (2002) Irritable pouch syndrome: a new category of diagnosis for symptomatic patients with ileal pouch-anal anastomosis. *Am J Gastroenterol* **97**: 972–977.

Stec R, Pławski A, Synowiec A, Mączewski M, Szczylik C (2010) Colorectal cancer in the course of familial adenomatous polyposis syndrome ("de novo" pathogenic mutation of APC gene): case report, review of the literature and genetic commentary. *Arch Med Sci* **6**: 283–287.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3**: RESEARCH0034.

Wang X, Spandidos A, Wang H, Seed B (2011) PrimerBank: a PCR primer database for quantitative gene expression analysis, 2012 update. *Nucleic Acids Research* **40**: D1144–D1149.

Yamamoto T, Umegae S, Kitagawa T, Matsumoto K (2005) The impact of the fecal stream and stasis on immunologic reactions in ileal pouch after restorative proctocolectomy for ulcerative colitis: a prospective, pilot study. *Am J Gastroenterol* **100**: 2248–2253.

Yanai H, Ben-Shachar S, Baram L, Elad H, Gitstein G, Brazowski E, Tulchinsky H, Pasmanik-Chor M, Dotan I (2014) Gene expression alterations in ulcerative colitis patients after restorative proctocolectomy extend to the small bowel proximal to the pouch. *Gut*; DOI: 10.1136/gutjnl-2014-307387.