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ORIGINAL ARTICLE

Effect of Long-Term Mesalamine Therapy on Cancer-Associated Gene Expression in Colonic Mucosa of Patients with Ulcerative Colitis

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Abstract

Background The role of 5-aminosalicylic acid (5-ASA or mesalamine) in the prevention of colorectal cancer in ulcerative colitis (UC) patients was reported, but the effect on molecular targets in UC colon mucosa is unknown.

Aim This observational study evaluates gene expression levels of 5-ASA targets using serial colon biopsy specimens from UC patients undergoing long-term 5-ASA therapy.

Methods Transcript levels were compared between colonoscopic biopsy specimens collected from 62 patients at initial and final follow-up colonoscopy at 2–6 years. All patients had mild-to-moderate UC and were undergoing long-term 5-ASA maintenance. Stepwise multiple linear regression analyses were performed to correlate changes in transcript levels with therapeutic response (Mayo clinical score endoscopy and DAI and/or Nancy histopathology score) and nonclinical variables.

Results The transcript levels of colorectal carcinogenesis-associated known 5-ASA target genes were significantly reduced after prolonged 5-ASA therapy ($P < 0.005$–0.03). Multiple linear regression models predicted significant association between transcript levels of Ki-67, NF-kB (p65), PPARγ, COX-2 and IL-8, CDC25A, and CXCL10 with duration of drug (5-ASA) exposure ($P \leq 0.05$). Ki-67, NF-kB (p65), and CXCL10 transcripts were also correlated with reduced endoscopy sub-score ($P \leq 0.05$). COX-2, IL-8, CDC25A, and TNF transcripts strongly correlated with DAI sub-scores ($P \leq 0.05$). Only COX-2 and IL-8 transcript levels correlated ($P \leq 0.05$) with Nancy histological score.

Conclusion This study provides molecular evidence of changes in carcinogenesis-related targets/pathways in colon tissue during long-term 5-ASA maintenance therapy that may contribute to the observed chemopreventive effects of 5-ASA in UC patients.

Keywords 5-aminosalicylic acid (5-ASA) · Chemoprevention · Ulcerative colitis (UC)

Introduction

5-Aminosalicylic acid (5-ASA, or mesalamine) is the mainstay drug for the treatment of acute colitis as well as for maintenance of remission in mild-to-moderate ulcerative colitis (UC). Epidemiologic studies suggest that UC patients have a 5.7 times higher relative chance of developing colorectal cancer (CRC) compared to the general population. This incidence rate corresponds to cumulative probabilities of CRC of 1.6% by 10 years, 8.3% by 20 years, and 18% by 30 years after disease diagnosis [1, 2]. Studies have shown a significantly reduced risk of CRC development in UC patients treated with 5-ASA or sulphasalazine [3–5]. 5-ASA treatment is associated with induction of apoptosis in CRC [6] patients. Oral administration of 5-ASA in human subjects with sporadic polyps was accompanied by an increase in the apoptotic rate and decrease in the proliferation of mucosal cells within days after the initiation of treatment [7]. A meta-analysis showed that the odds ratio of developing CRC in UC patients taking 1.2 g 5-ASA/day orally was 0.51 and decreased to 0.23 at higher doses [8]. Collectively, these findings indicate that chronic exposure to 5-ASA has anti-neoplastic effects. Despite several decades of research, the mechanisms of the therapeutic and chemopreventive actions of 5-ASA in UC remain unclear.

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In rodent models of CRC, 5-ASA has been shown to inhibit tumor growth and reduce the number of aberrant crypt foci [9]. When administered in the remission stage of colitis, it suppressed colitis-associated cancer in a murine model [10]. 5-ASA has demonstrated growth inhibitory and apoptosis-inducing effects in several different CRC cell lines in a time and dose-dependent manner [11–15]. Among the targets modulated by mesalamine reported in the literature, the most accepted are: *PPARγ* (HT-29 a CRC cell line) [16, 17] *NF-κB (CaCo2 and colon mucosa)* [18, 19], Wnt/β-catenin pathway (DLD-1, SW480 *and colon mucosa)* [14, 20–22], and *COX*-2 (HCT115 and HT-29) [14]. However, the precise molecular targets and pathways by which 5-ASA may modulate neoplastic progression are not fully understood [14, 23]. CRC cell lines deficient in or possessing a mutated form of *β-catenin* (HCT116 and SW48) or *COX-2* (DLD-1) also responded to 5-ASA exposure by undergoing either cell cycle arrest or apoptosis or reduced ability to form tumors, suggesting that alternative pathways are involved in the mechanisms of action of 5-ASA [14] and the anti-inflammatory effects of 5-ASA are congruent to anticarcinogenic effect in the cell culture models [24, 25]. In an earlier study, the transcripts of TC22 and a novel tropomyosin isoform associated with CRC [26] were suppressed by 5-ASA in the LS180 colon cancer cell line [27].

The potential anticancer effects of 5-ASA have been ascribed in part to a structural similarity with aspirin [7] and may be mediated by peroxisome proliferator-activated receptors (PPARγ) [16] and nuclear factor kappa B (NF-kB p65) [18]. Anticarcinogenic effects of 5-ASA have been clinically observed after prolonged therapy at a dose as low as 1.2 g 5-ASA per day, while higher doses (2.4–4.8 g per day) are effective for the induction and maintenance of remission in mild-to-moderate UC [1] [25]. Following ingestion, SASP and other 5-ASA azo compounds bypass small bowel absorption and are cleaved by the colonic bacterial azoreductase enzyme, allowing the 5-ASA moiety to achieve intraluminal concentrations in the colon in the range of approximately 7.0–14.0 mM [28]. Anticarcinogenic and antimicrobial activity of 2 mM and lower doses of 5-ASA have been demonstrated in vitro [27, 29].

In the present study, we evaluate the change in expression levels of carcinogenesis-related 5-ASA target genes selected from the literature (NF-kB p65, CXCL10, COX-2, TNF-α, IL8, PPAR-γ, CDC25A, Ki-67, BCL2L1, and β-catenin) [16–22, 26, 27], in colorectal tissues obtained from UC patients maintained on long-term 5-ASA therapy. These genes are also known to be associated with inflammation (NF-kB p65, CXCL10, COX-2, TNF-α, IL8, PPAR-γ) and cell cycle regulation (CDC25A, Ki-67, BCL2L1, and β-catenin). The correlation of transcript levels of target genes with clinical, endoscopic, and histopathological parameters of UC disease activity is investigated using multiple linear regression models to understand the molecular changes in the colonic tissue as a result of long-term 5-ASA use. These molecular changes confirm that indeed multiple redundant target pathways could be the basis for the therapeutic and chemoprophylactic effect of 5-ASA at clinically relevant concentrations.

Methods

Study Design

Colonoscopic biopsy specimens were collected in a prospective fashion after signed IRB-approved consent from patients with an established history of mild-to-moderate UC at the Crohn's and Colitis Center of New Jersey. The biopsy samples were included in the analysis if they fulfilled the following criteria: (a) age 18–80 years; (b) disease extent—proctosigmoiditis; (c) disease duration—greater than or equal to 5 years (exposure to 5-ASA was variable); (d) severity—mild-to-moderate disease; and (e) at least one follow-up visit in 2–6 years. Patients with the following characteristics were excluded: (a) severe UC or UC flare-up requiring hospitalization; (b) disease duration—fewer than 5 years; (c) proctitis only; (d) current corticosteroid treatment of 20 mg prednisone or greater. During this study, patients were treated with either 2.4 g or 4.8 g 5-ASA (mesalamine) per day. Mesalamine dose escalation during the course of the study was permitted at the clinical discretion of the treating physician (K.D.). Medication adherence was assessed at all clinical encounters utilizing a medication diary.

Colonoscopic Evaluation and Clinical Tissue Procurement

All patients consented to allocation of biopsy samples for RNA analyses in addition to histopathological analyses at the time of colonoscopy evaluation at the CCCNJ, subsequently at 1–2-year intervals as clinically indicated. Biopsies specimens were obtained from the rectosigmoid colon and allocated for formalin fixation for histopathological analysis or snap-frozen for RNA analysis. In order to compare gene expression among patients with differing UC disease extent, histopathology and RNA expression analyses reported in this study were only derived from rectosigmoid tissue biopsies. Snap-frozen biopsy specimens were stored at − 80 °C or in liquid nitrogen.

Baseline and Follow-Up Assessment of Clinical, Endoscopic, and Histologic Disease Activity

A clinical score (disease activity index, DAI) was assigned at the time of each endoscopy based on the Mayo UC

activity index clinical sub-score as follows: [30, 31] presence of blood in the stool (score 0–3); stool frequency [baseline number of bowel movement] (score 0–3); clinician assessment of disease activity (score 0–3). An endoscopy score was assigned for each colon segment and the rectum by the performing endoscopist (K.D.) based on the Mayo UC activity index endoscopy sub-score as follows: 0, normal or quiescent inflammation; 1, mild inflammation (altered vascular markings, erythema, edema); 2, moderate inflammation (absent vascular markings, friability, erosions); 3, severe inflammation (spontaneous bleeding, ulceration). A Nancy histopathology score [32] with the following numeric values: 0, no active inflammation; 1, mildly active inflammation; 2, moderately active inflammation; 3, severely active inflammation, was assigned to biopsy specimens in a blinded fashion by two gastrointestinal pathologists. Patients were considered to be in remission with DAI less than 1, and symptomatic for disease with a DAI greater than 1 at enrollment and follow-up evaluations. Patients treated with 5-ASA therapy were regarded as therapy "responders" if the endoscopy, DAI or histopathology scores decreased by at least one point from the initial colonoscopy to the final follow-up colonoscopy, or if an initial score of "0" remained unchanged. Patients with any initial score greater than "0" who did not show any reduction in any of the three scores, or whose Mayo clinical or histopathology scores increased with follow-up colonoscopies, were classified as "nonresponders."

Cell Culture

LS180 colon cancer cell lines (ATCC, Rockville, MD) were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM Glutamine, 100 u/ml Penicillin, and 0.01 mg/ml Streptomycin. A 20 mM stock solution of 5-ASA (A3537, Sigma-Aldrich, USA) was prepared fresh for each experiment, pH adjusted to 7.2, filtered, and diluted to a working concentration of 2 mM. For studying the effect of prolonged exposure to 2 mM drug, cells were treated for 24 h and then retreated with fresh drug solution every 24 h and then harvested at 72 h. The higher dose (20 mM) exposure was one time for 72 h. LS180 cells grown in parallel in serum-free DMEM without 5-ASA, served as controls. Cells were removed with 0.25% trypsin–EDTA, counted, and stained with trypan blue to verify that < 90% were viable in every experiment.

Microarray Bioinformatics Analysis

RNA was extracted from the LS180 cells exposed to 2 mM 5-ASA at 0, 4, and 24 h using the *Qiagen RNeasy Mini Kit* as per the manufacturer's instruction. The RNase-Free DNase set (Qiagen) was used during RNA purification, and cDNA was generated using *Advantage RT-for-PCR kit*. DNA contamination was tested by PCR of the RT samples. Double-stranded cDNA and biotin-labeled cRNA probes were made from 5 µg total RNA using the SuperScript Choice system (Invitrogen) and the ENZO BioArray, respectively. Procedures were performed according to recommendations from Affymetrix. This cRNA was hybridized to Hu-133A chips (Affymetrix) containing cDNA oligonucleotides representing 18,400 different transcripts from 14,500 genes followed by washing and staining on the GeneChip Fluidics Station 450 (Affymetrix) according to the manufacturer's instructions. The chips were scanned on the Affymetrix GeneArray® 2500 scanner. The quality of the RNA and probe was controlled by an Affymetrix-based test measuring the ratio between 5′ and 3′ mRNAs for β-actin and GAPDH and found to be highly satisfactory. The datasets originating from the duplicate samples for each time point were analyzed using the Affymetrix Mas5.0 software. Ingenuity Pathway Analysis (IPA) was used for identification of biological pathways and functions.

Cancer Pathway-Focused PCR Array

RT^{2}Profiler™ PCR Array, Human Cancer Pathway (SABiosciences, Frederick, MD, catalog# 330231), was utilized to analyze LS180 cells from multiple 5-ASA exposures experiments. RNA isolated from the cells was processed according to the manufacturer's instructions and run on ABI prism. This commercial, focused PCR array has primers from 84 known cancer-pathway-specific genes and housekeeping genes were included as internal controls and analyses tools were provided by the array kit manufacturer.

Real-Time RT-PCR

The genes and primers used for real-time RT-PCR are listed in Table 1. The *Roche Lightcycler* (Roche Applied Bioscience, Indianapolis, IN) and *Qiagen SYBR Green PCR Kit were used for real-time RT-PCR.* All samples underwent denaturing at 95 °C for 45 s, followed by 30 cycles of amplification at 62 °C for 10 s each and 72 °C for 12 s each. Samples were run in duplicate with test primers; actin was used as the internal control. Fold changes were calculated by normalizing the thresholds (*Ct*) of the test samples with the *Ct* values for actin (internal control) using the $\Delta\Delta Ct$ method.

Statistical Analysis

Analysis of variance (ANOVA) and Student's *t* test were used to investigate the effect of 5-ASA on individual gene expression changes between the initial and final biopsy samples. The threshold for a significant change in the microarray was set at a greater than twofold change

Table 1 Primers used for real-time RT-PCR

Genes	Primer sequence (5′–3′)
BCL2L1	
F	5′-TCCCTCGCTGCACAAATACTC-3′
R	5′-TTCTGCCCCTGCCAAATCT-3′
CDC25A	
F	5′-TAAGACCTGTATCTCGTGGCTG-3′
R	5′-CCCTGGTTCACTGCTATCTCT-3′
COX-2	
F	5′-CTGCAGAGTTGGAAGCACTCTA-3′
R	5′-CTTCCAGTAGGCAGGAGAACAT-3′
CXCL10	
F	5′-TTCAAGGAGTACCTCTCTCTAG-3′
R	5′-CTGGATTCAGACATCTCTTCTC-3′
CTNNB1	
F	5′-CCCACTGGCCTCTGATAAAGG-3′
R	5′-ACGCAAAGGTGCATGATTTG-3′
IL-8	
F	5′-TCTGGCAACCCTAGTCTGCT-3′
R	5′-GCTTCCACATGTCCTCACAA-3′
Ki-67	
F	5′-CTATGAGCCGGCTAAAATGAAG-3′
R	5′-GTTTTATCACCAGCCTTGAAGC-3′
NF-KB	
F	5′-CCACAAGACAGAAGCTGAAGTG-3′
R	5′-AGCCAGTGTTGTGATTGCTAGA-3′
PPAR-y	
F	5′-CCTGATAGGCCCCACTGTGT-3′
R	5′-CAGGTGGGAGTGGAACAAT-3′
P53	
F	5′-GGGAGTAGGACATACCAGCTTAGA-3′
R	5′-CTTCCCTGGTTAGTACGGTGAAGT-3′
TNF-alpha	
F	5′-GTAGCCCATGTTGTAGCAAACC-3′
R	5′-GACCTGGGAGTAGATGAGGTACAG-3′

compared with control ($P < 0.05$). Hierarchical clustering analysis was performed using a correlation metric and complete linkage (OmniViz Pro™, Maynard, MA). Multivariate linear regression analysis (stepwise regression, with Akaike information criterion (AIC) [33, 34] was used to determine the best set of variables (an optimal model) from a larger set of variants: age, sex, duration of disease, duration of drug exposure, concomitant use of 6MP, gene expression level at first endoscopy, Mayo clinical scores including endoscopy and DAI sub-score, and Nancy histopathology scores, that correlated with transcript levels. Only those variables with $P < 0.05$ or less were accepted for correlation with reduction in target gene expression levels.

Results

Ulcerative Colitis Patient Characteristics and Changes in Disease Status After Prolonged 5-ASA Therapy

The patient cohort that met criteria for inclusion in this study was comprised of 39 males and 23 females, age ranging from 23 to 68 years [median age of 43 years (range 23–68 years), 10 patients were older than 60 years]. The duration of disease in these individuals ranged from 5 to 38 years (median disease duration 11 years). A baseline colonoscopy and pathological analysis were performed immediately upon enrollment in the study. The mean Mayo score for the 62 patients was 5.6 indicative of mild-to-moderate disease. The disease extent was classified as pancolitis (inflammatory involvement of the entire colon) in 37 patients and left-sided colitis in 25 patients. Thirty patients were in remission (DAI ≤ 1) and 32 had active UC (DAI > 1) at enrollment. All patients were treated with mesalamine (2.4 g/day, $n = 34$; 4.8 g/day $n = 28$) during the entire period of observation (Table 2). None of the patients were treated with corticosteroids at enrollment, and 17 patients were taking 6-mercaptopurine (1–1.5 mg/kg body weight) in addition to 5-ASA.

The median interval between initial and final colonoscopy evaluations was 4 years (range 2–6 years). If the patient's condition so required, only their last colonoscopy evaluation before escalation from 5-ASA to biologics was retained for this study. At the term of this study, 49/62 (79%) of the patients were classified as responders to 5-ASA therapy. The Mayo clinical score of the cohort decreased by 28% (initial mean = 5.7 last/final mean = 4.0). The disease severity as observed during endoscopy decreased in 14/62 patients (22.6%), remained unchanged in 32/62 patients (51.6%), and increased in 16/62 (25.8%) patients from the initial to the final colonoscopy. The mean Nancy histopathology score of the cohort changed from 2.9 at enrollment to 1.56 at the term of the study, a 53% reduction in the scores indicating histologic improvement in response to 5-ASA. A total of 38/62 (59.7%) patients showed an improvement in histopathology, 17/62 (26.7%) remained unchanged, and 7/62 patients (11%) showed deterioration in histopathology (increased inflammation) during the course of observations in this study.

Table 2 Characteristics of the patient cohort

Characteristics of the patient cohort at enrollment colonoscopy	
Female	23
Male	39
Age	23–68 years[a]
Drug dose	
2.4 g per day	34
4.8 g per day	28
Duration of treatment	2–7 years
Duration of disease	5–38 years[b]
Extent of disease	
Left-sided colitis	25
Pancolitis	37
Mean Nancy histopathology scores	2.9
Mean Mayo clinic scores	5.6
Characteristics of the patient cohort at follow-up colonoscopy	
Duration of follow-up	2–6 years
Clinical remission	30
Symptomatic for disease	32
Mean Nancy histopathology scores	1.56
Mean Mayo clinic scores	4

[a]Median age 43 years; 21–40 years: 21; 41–60 years: 31; > 60 years: 10

[b]Mean duration of disease: 12 years; median 11 years

Transcript Levels of Selected Molecules Associated with Carcinogenesis and Inflammation Were Affected by Prolonged 5-ASA Therapy in UC Patients

The RNA expression levels of inflammation, cell cycle, and carcinogenesis-related genes in biopsy samples obtained during the initial and final colonoscopy are given in Table 3. The mean expression levels of 10 out of the 14 genes included in this study, including BCL2L1 (1.68-fold, $p < 0.03$), CEACAM-1 (1.29-fold, $p < 0.02$), CXCL10 (2.68-fold, $p < 0.017$), Cyclin D1 (1.74-fold, $p < 0.03$), Ki-67 (1.39-fold, $p < 0.03$), NF-κB (1.98-fold, $p < 0.005$), PPAR-γ (1.69-fold, $p < 0.01$), and P53 (1.61-fold, $p < 0.002$), were significantly lower in biopsy samples obtained during the final colonoscopy compared to those obtained during the initial colonoscopy. The expression levels of β-catenin, CDC25A, IL8, and TNF-α did not change significantly during the course of the study. Mean COX-2 gene expression was reduced 1.77-fold in biopsies from the final colonoscopy, but the change did not reach statistical significance.

Table 3 Change in transcript levels of carcinogenesis- and inflammation-associated target genes in colorectal biopsy tissues of UC patients after prolonged 5-ASA therapy

Genes	Fold change in transcript levels	*P* value
Carcinogenesis-associated genes		
Ki-67	− 1.39	0.034
CDC25A	− 1.2	0.1
CEACAM-1	− 1.29	0.022
BCL2L1	− 1.68	0.032
P53	− 1.61	0.002
CTNNB1	− 1.07	0.4
Inflammation- and carcinogenesis-associated genes		
CXCL10	− 2.68	0.017
COX-2	− 1.77	0.1
PPAR-γ	− 1.69	0.014
NF-κB	− 1.98	0.005
IL-8	− 1.18	0.5
TNFα	− 1.12	0.48

The fold changes were calculated based on transcript levels found in initial and final biopsy samples derived from all 62 patients

−, decreased expression

Correlation of Altered Transcript Levels with Clinical Parameters of Disease Activity in UC Patients Undergoing 5-ASA Therapy

Multiple linear regression models predicted that reduction in transcript levels of Ki-67, NF-kB (p65), PPARγ, COX-2 and IL-8, CDC25A, and CXCL10 was all significantly associated with duration of drug (5-ASA) ($P \leq 0.05$). While the reduced levels of Ki-67, NF-kB p65, and CXCL10 transcripts correlated with reduced endoscopy sub-score ($P \leq 0.05$), reduction in the transcript levels of COX-2, IL-8, CDC25A, and TNF transcript levels strongly correlated with reduced DAI sub-scores ($P \leq 0.05$). Only the COX-2 and IL-8 transcript levels correlated ($P \leq 0.05$) with Nancy histological score. The genes transcript levels were unaffected by dose of 5-ASA (2.4 g/day or 4.8 g/day) or the concomitant use of 6MP during therapy (Table 4).

Only 19 of the 62 patients (30%) had elevated levels of PPARγ transcripts in response to 5-ASA therapy, and 34/62 (54.8%) patients had lower transcript levels. While only 3 out of 19 (15.8%) patients with increased PPARγ expression showed a worsening of disease activity, 24/34 (74.6%) patients with a decrease in PPARγ expression showed an improvement as reflected by changes in the Mayo UC scores. Thus, explaining the lack of association between PPARγ transcripts and UC disease activity in the multiple regression model.

COX-2 expression was elevated in 18/62 patients (29%) and reduced in 30/62 (48.4%) patients. Five out of the 18 patients (27.8%) with increased COX-2 expression showed deterioration in disease, and 21/30 patients (70%) with reduced COX-2 expression showed improvement in the disease symptoms. NF-κB expression was elevated in 19/62 patients (30%) and suppressed in 34/62 (54.8%) patients. While 4/19 (21%) of the patients with increased NF-κB expression showed increased endoscopy sub-scores, 24/34 (70.6%) patients with reduced NF-κB expression showed reduction in endoscopy sub-scores. These observations corroborated association between COX-2 and NF-κB transcript levels and UC disease activity predicted by the multiple linear regression model.

Mesalamine Targets Several Anticarcinogenic Genes in the Absence of COX-2, PPARγ, or NF-kBp65

To confirm the above observations, that clinical response in UC patients could be achieved after 5-ASA therapy even without the participation of COX-2, PPARγ, or NF-kB p65, microarray was performed on the LS180 colorectal cells exposed to 2 mM 5-ASA for 4 and 24 h. The Hu-133A chips-based microarray identified a total of 241 genes that were down-regulated and 83 genes that were up-regulated after 4 h of 2 mM 5-ASA exposure. Clustering analysis of the microarray datasets obtained showed that these altered transcript levels returned to baseline at 24 h (Fig. 1a). Eight candidate genes were randomly selected for confirmatory RT-PCR analysis for both time points (4 and 24 h), and all the genes tested demonstrated a similar trend when compared with the microarray (Fig. 1b).

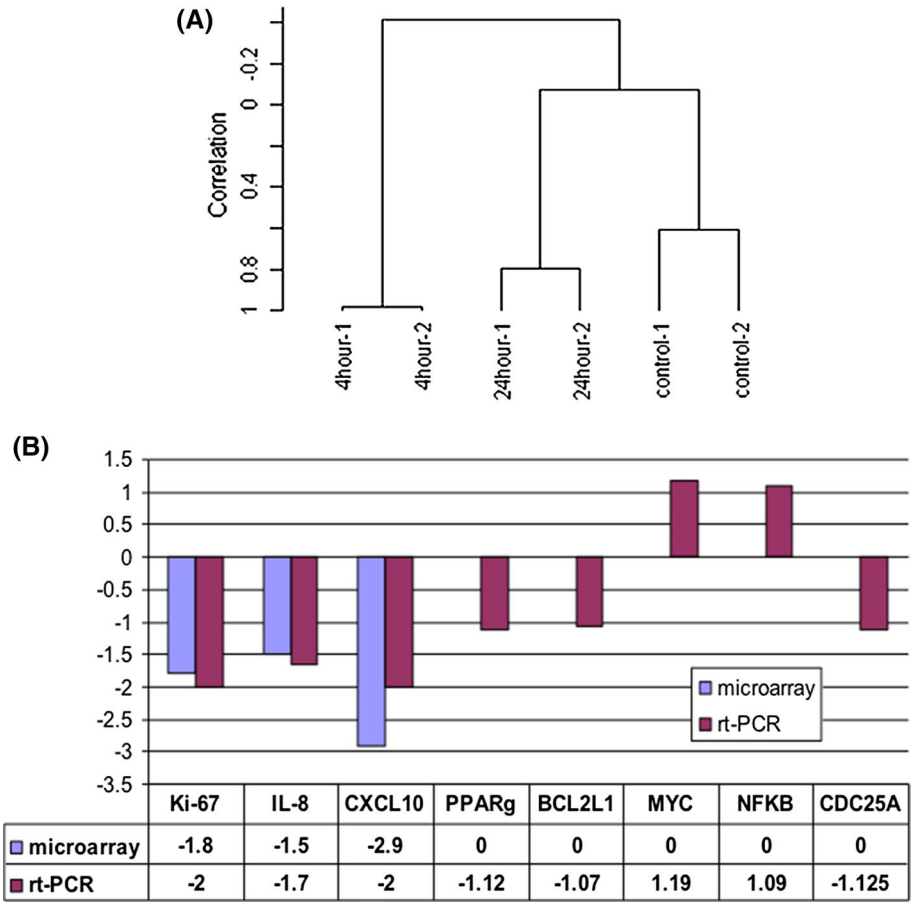
Ingenuity Pathway Analysis of the microarray datasets revealed that the targets affected by 5-ASA exposure were primarily associated with "Cancer" and "Gastrointestinal disease." The molecules belonged to gene networks with major biological functions related to cell cycle progression, cellular growth and proliferation, carcinogenesis, inflammatory response, and DNA replication (supplementary data). The Ingenuity Knowledge Base also revealed that genes altered by 5-ASA in this microarray dataset functionally identified with canonical pathways associated with DNA damage response, cell cycle checkpoint control, mitosis, and cancer signaling (Supplementary figures S1–S3).

When LS180 cells were exposed to fresh 2 mM 5-ASA every 24 h for 72 h, the transcript levels of the carcinogenic genes did not revert back to baseline but persisted at the altered levels seen at 4 h. Human cancer-pathway-specific RT^{2}Profiler™ PCR Array identified the target genes related to cell survival (BCL2L1, −2.9-fold at 48 h, −5.2 at 72 h,

Table 4 Independent variables that correlate* with transcript levels of 5-ASA target genes in the UC patients

Gene targets	TC22	Ki-67	NF-KB	PPARγ	COX-2	IL-8	CXCL10	CDC25A	TNFα
Variables correlating with change in transcript levels									
Duration of drug exposure	$P=0.002$	$P=0.001$	$P=0.017$	$P=0.004$	$P=0.009$	$P=0.04$	$P=0.0003$	$P=0.01$	
Endoscopy sub-score		$P=0.056$	$P=0.05$				$P=0.008$		
DAI sub-score					$P=0.006$	$P=0.03$		$P=0.01$	$P=0.004$
Nancy histopathology scores					$P=0.007$	$P=0.008$			

*The correlation was established using multiple linear regression analyses, which included other independent variables: age, sex, duration of disease, concomitant use of 6MP, gene expression level at first endoscopy. Only those variables that were significantly ($P<0.05$) associated with change in transcript levels of at least one gene are listed on the table



	Ki-67	IL-8	CXCL10	PPARg	BCL2L1	MYC	NFKB	CDC25A
microarray	-1.8	-1.5	-2.9	0	0	0	0	0
rt-PCR	-2	-1.7	-2	-1.12	-1.07	1.19	1.09	-1.125

Fig. 1 **a** Clustering analysis of the microarray datasets from LS180 CRC cell line exposed to 2 mM 5-ASA for 0, 4, and 24 h. **b** Confirmation of microarray data of randomly selected genes using real-time RT-PCR

$p < 0.01$), cell cycle progression (CDC25A, − 1.9-fold at 48 h, − 2.1 at 72 h, $p < 0.01$), and inflammation (IL8, − 5.7-fold at 48 h, − 7-fold at 72 h, $p < 0.01$). Increased expression of the apoptotic marker Caspase 8 (CASP8) was noted only at 48 and 72 h but not at 4 h (Table 5). PPARγ, COX-2, and NF-kB p65 remained unchanged at all times.

Discussion

While the anticarcinogenic effects of sulfasalazine and 5-ASA in UC have been suggested by clinical studies, the molecular mechanisms of these effects remain to be determined. The aim of this study was to measure the effect of long-term 5-ASA therapy on carcinogenesis-related gene expression in colon biopsy tissues from UC patients, and thus provide insights into the molecular basis of the observed chemoprophylactic effects of 5-ASA. The genes for NF-kB (p65), PPAR-γ, COX-2, CXCL10, TNF-α, IL8, CDC25A, β-catenin, and p53 have been reported to participate in mediating the growth inhibitory, pro-apoptotic, antioxidative, antimicrobial, and anti-inflammatory effects of 5-ASA on colorectal cells [13, 14, 16, 18, 20, 26, 35–38]. Other target genes, such as Ki-67, a proliferation marker also

Table 5 Transcript levels of carcinogenesis- and inflammation-associated target genes in LS180 cells exposed to 2 mM mesalamine for 4, 24, 48, and 72 h*

Exposure time	2 mM-4 h	2 mM-24 h	2 mM-48 h	2 mM-72 h
Gene targets		5-ASA replenished every 24 h		
Carcinogenesis-associated genes				
Ki-67	− 1.8	1.2	− 1.8	− 1.6
CTNNB1	− 2.08	− 1.19	− 2.1	− 2.15
CASP8	− 1.02	− 0.2	1.8	2.2
BCL2L1	− 1.07	− 1.21	− 2.9	− 5.2
CDC25A	− 1.1	− 0.9	− 1.9	− 2.1
Inflammation- and carcinogenesis-associated genes				
CXCL10	− 2	1.3	− 3.2	− 3.5
IL-8	− 1.5	1.3	− 5.7	− 7
PPARγ	− 1.12	− 1.2	− 1.2	− 1.14
COX-2	− 1.16	− 1.03	− 0.91	− 0.9
NF-kB	1.09	− 1.1	− 1.03	− 0.98

*2 mM mesalamine exposure was repeated every 24 h; thus the cells harvested at 48 h were exposed to 2 mM 5-ASA twice, once at 0 h and again at 24 h. The minus sign represents fold suppression of gene expression compared to untreated control (0 h) cells

Changes represent average fold changes from three sets of independent observations ($P < 0.05$)

used as a prognostic marker in cancers [39], BCL2L1, an important apoptosis regulating gene that codes for both an anti-apoptotic Bcl-xL and a pro-apoptotic Bcl-xS splice variants [40], carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM-1), a cell adhesion molecule with a wide range of biological functions regulating cell signaling and considered as a therapeutic target in cancer [41], and P53 (a key protein involved in cell cycle checkpoint regulation) [42], were identified in this study after preliminary microarray and pathway-specific RT^{2}Profiler™ PCR array performed on LS180 CRC cell line exposed to 5-ASA.

The reduction in transcript levels of COX-2, CDC25A, CXCL10, IL8, NF-κB, and Ki-67 in colonic biopsy tissues was found to correlate with indices of disease severity and duration of 5-ASA therapy in this UC patient cohort. These anti-inflammatory and cell cycle regulatory markers may be interpreted as reporting on the anticarcinogenic actions of 5-ASA and disease activity in general. The anti-inflammatory effects of 5-ASA may certainly overlap with its anticancer effects. Indeed, UC disease severity always associated with inflammation is a known risk factor for colitis-associated dysplasia and colorectal cancer. However, it is noteworthy that reductions in the expression of some genes (PPARγ, CEACAM, β-catenin, and P53) that are known to be involved in the carcinogenesis process did not correlate with UC disease activity in these patients. Also, COX-2, IL-8, and TNF known to be important landmarks of inflammation were found to correlate with clinical presentation of inflammation (DAI and histology), whereas Ki-67, NF-kB, and CXCL10 associated with both inflammation and carcinogenesis were correlated with endoscopy only. This may suggest a chemopreventive effect even in the absence of a perceptible improvement in UC disease activity. It may also be prudent to consider that the biopsy samples represent a mixed cellular population, including epithelial cells, mesenchymal cells, and inflammatory cells; it is challenging to differentiate anti-inflammatory from anticarcinogenic effects based on changes in gene expression alone. Future efforts assessing the in situ expression of these genes or their protein levels in biopsies obtained from 5-ASA-treated patients would provide additional insights into the role of these molecular targets of 5-ASA.

In colorectal cancer cell lines and murine models of UC, the anti-inflammatory and antineoplastic effects exerted by 5-ASA exposure were accompanied by increased levels of PPARγ and, in some cases, simultaneous lowering of COX-2 expression [16, 17, 43]. It has been reported that UC patients have significantly lower levels of PPARγ compared to healthy volunteers, [35] and patients with active UC treated with topical rosiglitazone (a PPARγ agonist) exhibited reductions in the Mayo endoscopy sub-scores to a similar degree as 5-ASA [44]. In the present study, however, transcripts levels of PPARγ did not correlate with duration of exposure or dose of 5-ASA (2.4 mg or 4.8 mg), disease activity, or the duration of disease. The COX-2 expression, however, correlated significantly with histopathology, DAI, duration of disease, and duration of 5-ASA exposures. Therefore, we used the LS180 cells to understand the 5-ASA effect in the absence of PPAR-γ, NF-κB, and COX-2 implicated in the literature as mesalamine targets [16–22]. The LS180 cells lack COX-2 protein expression [45] and PPARγ although constitutively expressed it is not inducible with pioglitazone or its agonists in this cell line or its variant LS174T [46]. Yet, microarray analysis of the LS180 cells exposed to 5-ASA and subsequent *Ingenuity Pathway Analysis* did demonstrate, a priori, that 5-ASA modulated molecules primarily associated with gastrointestinal diseases and cancer. Recurring exposure to 2 mM 5-ASA every 24 h sustained suppression of gene targets (Ki-67, CXCL10, IL-8, BCL2L1, CASP8 and CDC25A) involved in inflammation, cell cycle checkpoint regulation, and apoptosis. The phenomenon of S-phase accumulation reported earlier in HT29 cells with 20 mM 5-ASA [11] was achievable after three consecutive exposures to 2 mM 5-ASA every 24 h (data not presented). Apoptosis was not observed in LS180 cells; however, increased Caspase 8 at 48 h (after retreatment with 5-ASA) may indicate sensitization of cells toward Fas-mediated cell death [47] as previously reported in other colorectal cancer cell lines [12, 15]. These observations indicate that adherence to a long-term 5-ASA regimen is required for maximum therapeutic success.

The discrepancy in the effects of 5-ASA on these genes between studies may be related to differences in route of drug administration (topical enema vs. oral), 5-ASA dosage, duration of exposure, and timing of exposure (i.e., during the period of acute inflammation, vs. during the period of chronic inactive, or quiescent, disease). These observations also confirm that the chemoprophylactic effects of 5-ASA are exerted via multiple and likely redundant target pathways. Although increased levels of COX-2 have been implicated in CRC and IBD [48, 49] and suppression of COX-2 by 5-ASA [43] or selective inhibitors [50] led to apoptosis of CRC cells, the growth inhibitory effects of 5-ASA have also been demonstrated independently of the COX-2 pathway in the CRC cell lines [14].

These results must be considered in the context of the study limitations. While the tissue samples were obtained in a prospective fashion, they were nevertheless collected during the course of "real-world" practice. The patients were not randomized based on 5-ASA dose, and treatment escalation was permitted at the discretion of the treating physician, which likely interfered with the ability to discern a dose-dependent response to 5-ASA. This study also lacked placebo and non-disease control groups, and no corrections could be made for any change in food habits or smoking. The patients included in this study suffered from

mild to moderate UC and were almost universally treated with 5-ASA or SASP as first-line therapy before being referred to the Crohn's and Colitis Center. Since all of the patients had a diagnosis of UC for at least 5 years at study inclusion, it was typically not possible to procure tissue samples from a 5-ASA naïve population. However, since the tissue samples were procured in a prospective fashion, biopsy samples from the patient's last follow-up visit (at the term of this study) were analyzed to observe the effects of long-term 5-ASA treatment on tissue-level gene expression in individual patients. This study did not investigate the effects of 5-ASA on dysplasia or colorectal cancer incidence in this patient cohort. Recent population-based studies have shown that the incidences of these lesions are markedly lower than those reported in seminal studies published three to four decades ago [1–4, 51] Therefore, a study of the chemopreventive effects of 5-ASA using such histopathological endpoints would require prohibitively large patient numbers followed prospectively for a longer duration. However, the strengths of this study include the use of in vitro models to complement studies in human tissues investigating mechanistically plausible gene pathways and molecular evidence to explain the lower incidence of colorectal cancer in patients on long-term 5-ASA. Furthermore, this study design provided a unique opportunity to compare the tissue-level gene expression profiles longitudinally from the same cohort of patients that responded to prolonged 5-ASA therapy.

These results indicate that several genes related to carcinogenesis are modulated by 5-ASA at clinically relevant concentrations in colorectal tissues, thus providing a basis for its epidemiologically observed chemotherapeutic effect in UC patients. It is intriguing to note that some of the molecular changes noted in colorectal tissues from UC patients undergoing 5-ASA therapy did not necessarily correlate with parameters of disease severity, which may provide a rationale for long-term use of 5-ASA as colorectal cancer chemoprophylaxis in UC patients, including those requiring immunosuppressive therapy.

Author's contribution MB participated in study design, data acquisition, data analysis, writing the first draft, and coordinating the subsequent revisions of the manuscript with coauthors. DS, KMD, and EP participated in patient recruitment; MB, DS, JG, XG, JA, CM, KD, and PA assisted in data collection from experiments and patient charts; SG and JC assisted in statistical analysis; MB, DS, and KMD contributed to critical review of the draft, and all authors reviewed and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest KD, XG and MB received funding for this study from Proctor and Gamble. Other authors have no conflicts to disclose.

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