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OBSERVATIONAL STUDY

Relationship between methylation and colonic inflammation in inflammatory bowel disease

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Abstract

AIM: To investigate the relationship between the methylation status in the *SLIT2* and *TGFB2* promoters and colonic inflammation in inflammatory bowel disease patients.

METHODS: We evaluated the methylation status of 2 genes (*SLIT2* and *TGFB2*) in 226 biopsies taken from 62 colonoscopies of 38 patients (29 ulcerative colitis and 9 Crohn's colitis) using methylation-specific melting curve analysis. The relationships between methylation status and clinical, biological, endoscopic and histological activities were evaluated. Twenty-three of the 38 patients had a second colonoscopy and were included in a longitudinal analysis. Numerical results were given as the means ± SD of the sample and range, except when specified. Student *t* analysis, *U* Mann Whitney and ANOVA factor were used to compare the means. Qualitative results were based on the χ^2 test.

RESULTS: *SLIT2* methylation was more frequent in samples with endoscopic activity than with endoscopic remission (55% *vs* 18%, $P < 0.001$). *SLIT2* methylation was also higher in samples with acute inflammation (56.5%) than in samples with chronic (24%) or absent inflammation (15%) ($P < 0.001$). For *TGFB2* methylation, the correlation was only significant with endoscopic activity. Methylation was higher in the distal colon for both genes ($P < 0.001$ for *SLIT2* and $P = 0.022$ for *TGFB2*). In the multivariate analysis, only inflammation status (and not disease duration or extension) was independently associated with *SLIT2* methylation [OR = 6.6 (95%CI: 1.65-27.36), $P = 0.009$]. In the longitudinal analysis, the maintenance of endoscopic remission was protective for methylation.

CONCLUSION: Endoscopic and histological inflammation are predictive for *SLIT2* methylation.

Key words: Colonic inflammation; Inflammatory bowel disease; Aberrant methylation; Dysplasia; Colitis associated colorectal cancer

Core tip: In this paper, we analyze the relationship between the methylation status of selected genes (*TGFB2*, *SLIT2*) and the inflammation status (according to endoscopic and histological activity) in ulcerative colitis and Crohn's colitis patients with increased risks for colorectal cancer. We observed that methylation correlated better with histological activity than with endoscopic activity. *SLIT2* and *TGFB2* were more frequently methylated in the distal colon. In the multivariate analysis after adjusting for disease extension, disease duration and inflammatory status, only inflammatory status was an independent predictor of methylation. We also observed that the maintenance of histological healing with time might be protective for methylation.

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INTRODUCTION

Dysplasia and Colitis-associated Colorectal cancer (CAC) are among the main complications of chronic ulcerative colitis (UC) or Crohn's colitis (CC) patients, although in recent years, population studies have shown their incidence to be reduced[1,2]. The diagnosis and prevention of dysplasia and CAC in patients with inflammatory bowel disease (IBD) remain a challenge[3,4]. When available, chromoendoscopy with targeted biopsies is the technique of choice to perform the colonic surveillance for dysplasia in IBD patients[5,6]. The histological diagnosis of dysplasia is hampered by significant inter-observer variations linked to concurrent chronic inflammatory changes and the specific histological features of dysplastic lesions[7,8]. Chronic intestinal inflammation has been associated with an increased risk of CAC[3,4,9-11]. Both the presence and the severity of such histological inflammation are independent risk factors for dysplasia/CAC in IBD patients[12] and can be even more predictive than endoscopic activity[11]. However, their clinical performance is still limited.

Biomarkers capable of stratifying patients according to their risk are greatly needed to optimize surveillance programs[13]. Three molecular pathways, according to the type of genetic alterations detected (chromosomal instability, microsatellite instability and methylator pathway), have been described in sporadic CRC. These pathways are also present in CAC at different times and frequencies[3,4,14]. The aberrant methylation of specific gene promoters is a common event in CAC, although its relative contribution is a matter of controversy[3,4,15-18]. Because methylation often precedes dysplasia and CAC[10,19], these molecular changes may be used as surrogate biomarkers for IBD patients with an increased risk of dysplasia or CAC.

The molecular and histological pathways leading to CAC in chronic IBD are still relatively unknown. The relationship between inflammation and methylation has been already studied in other organs, such as *Helicobacter pylori* infections in the stomach[20,21], chronic biliary tract inflammation[22] and Barrett's esophagus[23]. In IBD, some studies have already suggested that aberrant methylation might be related with the development of dysplasia and CAC[24,25]. On the other hand, inflammation has been associated with a higher methylation rate in IBD[25,26].

In a previous study, we showed that the *SLIT2* and *TGFB2* promoters offered a better discrimination between tumorous and adjacent mucosa in CAC, showing distinct patterns in the patients at increased risks or low risks of developing dysplasia or cancer[27]. However, a number of issues remain to be elucidated. While ulcerative colitis-CAC is more frequent in the distal colon[28-30], no data are available on methylation, according to colonic location. Furthermore, little is known regarding the persistence of methylation when the inflammation status changes over time.

The aim of our study was to explore the relationship between the methylation status in the *SLIT2* and *TGFB2* promoters and the histological and endoscopic activities in IBD patients at an increased risk for neoplasia. Additionally, we assessed the impact of colonic location and performed a preliminary longitudinal analysis of methylation status according to histological and endoscopic inflammation.

MATERIALS AND METHODS

Patients and tissue samples

Patients with an appropriate diagnosis of UC or CC who were considered to be at an increased risk for dysplasia and CAC were consecutively included. An increased risk for dysplasia and CAC was defined as UC affecting colonic mucosa proximal to rectum or CC affecting more than one-third of the colon. In both cases, the duration of the disease was > 8 years[31,32]. Patients with CD of ileal or ileocolonic locations were excluded.

A total of 62 colonoscopies were performed on 38 IBD patients (29 UC and 9 CC) between December 2010 and June 2012. Fifteen of these 38 patients had a single colonoscopy. A second colonoscopy was performed in 23 patients, who were included in a longitudinal analysis. Of the 62 colonoscopies, 57 were completed with indications of dysplasia surveillance, and samples were taken from the five segments of the colon (rectum, left colon, transverse colon, right colon and cecum). Five rectosig-

Table 1 Patient characteristics *n* (%)

Patient characteristics	Value
Number of patients	38
Patients with ulcerative colitis (UC)/Crohn's colitis (CC)	29 (76)/9 (24)
Men	23 (58)
Age (yr): mean ± SD	55.24 ± 14.11
Disease duration (yr): mean ± SD	17.82 ± 8.29
Number of colonoscopies	62
Number of colonoscopies in UC/CC	49 (79)/13 (21)
Disease extension[1]	
Left colitis (UC) or segmentary colitis (CC)	20 (33)
Extensive colitis (UC and CC)	42 (68)
Medication at endoscopy[1]	
Oral 5-ASA	40 (65)
IMM (AZA/6MP)	18 (29)/3 (5)
TNF inhibitor (IFX/ADA)	2 (3)/2 (3)
CRP (mg/dL): mean ± SD	8.25 ± 16.56
Fecal calprotectin (μg/g): mean ± SD	306.5 ± 690.1
Reason for colonoscopy	
Endoscopic activity assessment	5 (8)
Dysplasia surveillance	57 (92)
Clinical activity	
Active disease	5 (8)
Symptomatic remission	57 (92)
Endoscopic activity	
Endoscopic remission	24 (39)
Endoscopic activity	38 (61)
Histological activity and presence of dysplasia	
Dysplasia	8 (13)
Acute histological activity	23 (37)
Chronic histological activity	24 (39)
No histological activity	7 (11)
Methylation status	
SLIT2 (patients with any methylated sample)	40 (65)
TGFB2 (patients with any methylated sample)	23 (42)

[1]As therapy regimens overlapped, the total is 104.7%. ASA: Aminosalicylic acid; CS: Corticosteroids; IMM: Immunomodulators; AZA: Azathioprine; 6MP: 6 mercaptopurine; MTX: Methotrexate; TNF: Tumor necrosis factor; IFX: Infliximab; ADA: Adalimumab.

moidoscopies were performed, and in these cases, only samples of the rectum and sigmoid colon were taken. Rectosigmoidoscopies were performed when there was no indication for dysplasia surveillance. In all, 226 colonic biopsies were taken. Most of the patients were in clinical remission, and the reason for the colonoscopy was surveillance for dysplasia. Only 5 patients underwent colonoscopies due to clinical activity or to check their endoscopic remission (ER). The epidemiological, clinical, biological and endoscopic characteristics of the patients are shown in Table 1.

Disease activity

Clinical activity: Clinical activity was scored according to the Mayo index of the UC and Crohn's disease (CD) activity index (CDAI) for CC. For UC patients, symptomatic remission was defined as a Mayo stool frequency subscore of 0 or 1 and a Mayo rectal bleeding subscore of 0, and for CD patients, as CDAI < 150. Biological activity was measured according to CRP and fecal calprotectin (FC) levels. A CRP < 5 mg/L and a FC < 250 μg/g were considered "normal". In a previous study[33], we showed that a 250 μg/g cut-off value for UC was the most accurate cut-off level for ER.

Endoscopic activity: Colonoscopies were performed by 2 experienced gastroenterologists (Rodríguez-Moranta F, Guardiola J). Endoscopic activity was scored according to the Mayo endoscopic subscores for UC patients and to the CD activity index of severity (CDEIS) for CD patients. "Endoscopic activity" was defined as Mayo > 0 for UC or CDEIS > 0 for CD. "ER" was defined as Mayo = 0 for UC or CDEIS = 0 for CD.

Histological activity: Histological activity was assessed by 2 experienced pathologists (Sanjuan X, Loayza C). "Active disease" was defined by the presence of polymorphonuclear cells in conjunction with epithelial cell damage. "Inactive chronic disease" was defined by architectural changes (irregular surface and crypt abnormalities) and an increase in lamina propria mononuclear cells. "Quiescent disease" was reflected by the presence of architectural changes without alterations in the intensity or composition of the lamina propria cellular infiltrate[34].

Methylation-specific melting curve analysis of SLIT2 and TGFB2 gene promoters

DNA from the biopsies was extracted using a phenol chloroform method[35,36]. DNA (750 ng) was chemically modified to convert all un-methylated cytosines to uracils using the EZ DNA Methylation-Gold Kit® (Zymo Research, Orange, CA), according to the manufacturer's protocol. The methylation status of the *SLIT2* and *TGFB2* gene promoters was assessed by methylation-specific melting curve analysis (MS-MCA)[27,37]. Briefly, 50 ng of bisulfite-treated DNA was used as a template for the polymerase chain reaction (PCR) for 35 cycles of 10 s at 95 ℃, 20 s at the corresponding annealing temperature, and 25 s at 72 ℃, including a final extension step using the LightCycler 480 (Roche Applied Science, Indianapolis, IN) in the presence of the Fast Start DNA Master Sybr Green I mix (Roche Applied Science). Melting curves of the PCR fragments were analyzed in real time, as described. Samples were scored using LightCycler 480 Software v. 1.5 and classified as un-methylated or methylated to compare the melting curve positions of test samples with controls. The analytical sensitivity of the method varies between 1%-5%[27].

Ethical considerations

The ethics committee of the Bellvitge University Hospital approved this study, and all patients gave their informed written consent for participation.

Statistical analysis

Numerical results were given as the means ± SD of the sample and range, except when specified. *T*-Student analysis, *U* Mann Whitney and ANOVA factor were used to compare the means. Qualitative results were based on the χ^2 test. All *P* values were two-sided. $P < 0.05$ was consid-

Table 2 Relationship between methylation status and disease activity *n* (%)

Relationship	*SLIT2* methylation (yes/no)	*TFGB2* methylation (yes/no)
Endoscopic activity[1]		
Activity	38/31 (55)	22/41 (35)
Remission	28/129 (18)	18/127 (12)
P value	< 0.001	< 0.001
Histological activity		
Acute	26/20 (57)	7/29 (20)
Chronic	25/80 (24)	23/82 (22)
No activity	10/55 (15)	5/52 (9)
P value	< 0.001	NS
Clinical activity[2]		
Active	5/0 (100)	2/2 (50)
Remission	35/22 (61)	21/30 (41)
P value	0.151	NS
Biological activity		
Active (CRP ⩾ 5 mg/L + FC ⩾ 250 μg/g)	7/0 (100)	5/2 (71)
Remission (CRP < 5 mg/L + FC < 250 μg/g)	12/7 (63)	4/13 (24)
P value	0.134	0.061

[1]Endoscopic activity was defined as Mayo > 0 for ulcerative colitis (UC) or Crohn's disease (CD) activity index of severity (CDEIS) > 0 for CD. Endoscopic remission was defined as Mayo = 0 for UC or CDEIS = 0 for CD; [2]Clinical activity, for UC patients symptomatic remission was defined as a Mayo stool frequency subscore of 0 or 1 and a Mayo rectal bleeding subscore of 0; and for CD patients as CD activity index < 150. CRP: C-reactive protein; FC: Faecal calprotectin; NS: Not significant.

Table 3 Methylation status and disease activity according to disease location

Variable (% samples)	Distal colon	Proximal colon	*P* value
Endoscopic activity[1]	48/101 (48)	21/121 (17)	< 0.001
Active histological activity[2]	30/97 (31)	16/115 (13)	0.004
Methylation of *SLIT*[2]	43/101 (43)	23/121 (19)	< 0.001
Methylation of *TGFB*[2]	24/90 (27)	16/117 (14)	0.022

[1]Endoscopic activity was defined as Mayo > 0 for ulcerative colitis or Crohn's disease activity index of severity > 0 for Crohn's disease; [2]Active histological activity was defined as the presence of neutrophils in conjunction with epithelial cell damage.

ered statistically significant. Multivariate analysis consisted of logistic regression. The statistical analysis was carried out using the SPSS version 19.0 statistical package (SPSS Inc., Chicago, IL, United States).

RESULTS

Relationship between methylation status and clinical, biological, endoscopic and histological activity

First, we wanted to assess whether the methylation status was correlated with the known clinico-pathological variables used to score disease activity in a transversal analysis. *SLIT2* methylation was more frequent in samples with endoscopic activity, found in 38 of 69 (55%) endoscopically active *vs* 28 of 157 (18%) endoscopically inactive ($P < 0.001$). *SLIT2* methylation also correlated with histological inflammation, as its levels were higher in samples with acute inflammation (57%) than in samples with chronic (24%) or absent inflammation (15%) ($P < 0.001$). For *TGFB2* methylation, the correlation was only significant between methylation and endoscopic activity, likely due to the lower prevalence of methylation observed for *TGFB2* (Table 2). There was no correlation between *SLIT2* or *TGFB2* methylation status and clinical or biological activity. Of note, most of the patients included in this study were in clinical and biological remission (Table 2).

Table 4 Histological activity and disease location as predictors of *SLIT2* and *TGFB2* methylation status

Multivariate analysis	Distal location	Acute histological activity[1]
SLIT2		
Coefficient (β)	0.95	1.45
OR (95%CI)	2.57 (1.34-4.99)	4.25 (2.07-8.72)
P value	0.005	< 0.001
TGFB2		
Coefficient (β)	0.95	0.002
OR (95%CI)	2.59 (1.21-5.54)	1.01 (0.39-2.57)
P value	0.014	0.996

[1]Acute histological activity was defined as the presence of neutrophils in conjunction with epithelial cell damage.

To further explore the correlations with endoscopic and histological activity and methylation, the subset of patients with ER was analyzed. Seven of the 15 (47%) patients with ER and chronic inflammation had at least one of the 2 promoters of the genes methylated.

Methylation status, disease location and inflammatory status

A clear correlation was observed between disease location and inflammatory status (endoscopic and/or histological). A higher inflammatory status in the distal disease correlated with a higher prevalence of *SLIT2* methylation (43 % *vs* 19%, respectively, $P < 0.001$). Similar results were obtained for *TGFB2* methylation (27% *vs* 14%, respectively, $P = 0.022$) (Table 3). In the multivariate analysis, both the distal location and histological activity were independent predictors for *SLIT2* methylation. However, *TGFB2* methylation was only independently related to distal location (Table 4). Samples with dysplasia were significantly more methylated than those without dysplasia (80% *vs* 27.9%, $P = 0.027$), although this result should be carefully interpreted due to the small number of events. Furthermore, 3/5 samples with dysplasia were present in patients who had some grade of histological inflammation.

Disease extension, disease duration and inflammatory status are common criteria to consider for a patient at increased risk for neoplasia. We wondered whether these variables were also predictors of methylation. In the multivariate analysis, only the inflammatory status (endoscopic and/or histological) was an independent predictor of *SLIT2* methylation: OR = 6.6 (95%CI: 1.65-27.36),

Table 5 Changes in inflammatory status and *SLIT2* methylation from colonoscopy 1 to colonoscopy 2

Changes in methylation of *SLIT2*		Changes in endoscopic inflammation				
		Stable (n = 50)		Change (n = 16)		
		ER→ER	Active→active	ER→active	Active→ER	Total
Stable	UM → UM	25	6	2	1	34
(n = 44)	M → M	2	5	0	3	10
Change	UM → M	2	2	7	0	11
(n = 22)	M → UM	7	1	3	0	11
Total		36	14	12	4	66

ER: Endoscopic remission; Active: Endoscopic activity; UM: Unmethylated; M: Methylated.

P = 0.009. In contrast, no correlation was evident for *TGFB2* methylation (data not shown). No differences were found in the methylation status of either of the 2 genes, according to the different medical treatments (data not shown).

Longitudinal analysis

Next, we explored whether longitudinal changes in the inflammatory status of the colonic mucosa were accompanied by changes in *SLIT2* methylation in the 23 UC patients who underwent a second colonoscopy within a one-year period. The results are shown in Table 5. The median time between the 2 colonoscopies was 238 d (range: 98-366 d). The most prevalent situation was the maintenance of endoscopic remission in both colonoscopies. Among the 48 segments with endoscopic remission in the 1st colonoscopy, 36 remained without endoscopic activity in the 2nd one. In this situation, the methylation status of *SLIT2* decreased from 25% (9/36) to 11% (4/36) (P = 0.22). Considering the segments that remained endoscopically active in both explorations (14 out of the 15 segments from the 1st colonoscopy), the prevalence of *SLIT2* methylation remained similar, from 43% (6/14) to 50% (7/14), (P = 1).

In those segments with endoscopic activity in the 1st colonoscopy that became endoscopic remission in the 2nd colonoscopy, the *SLIT2* methylation did not change (3/4, 75% in both cases, P = 1). Finally, in the 12 biopsied segments with endoscopic remission in the 1st colonoscopy that presented endoscopic activity in the second one, a non-significant trend towards an increase in *SLIT2* methylation was observed, from 25% (3/12) to 58% (7/12), P = 0.28.

DISCUSSION

In this study, we have shown a strong correlation between *SLIT2* methylation status and inflammation, according to the endoscopic and histological activities in colonic biopsies from IBD patients at increased risk for dysplasia and CAC. Microscopic inflammation was a more accurate marker for predicting methylation than endoscopic activity. *SLIT2* and *TGFB2* were more frequently methylated in the distal colon, but only *SLIT2* was independently associated with inflammation status. Changes in the methylation status occurred during follow-up, and the appearance of endoscopic activity was linked with an increased methylation status rate with time, whereas the maintenance of endoscopic remission was protective for this status. However, further longitudinal studies are needed to clarify whether *de novo* methylation correlates with inflammatory activity.

Inflammation is known to be a risk factor for CAC in these patients[3,4,9-11]. However, data on the relationship between inflammation and methylation in CAC among IBD patients are scarce. Saito *et al*[26] previously showed that the methylation of *CDH1*, *GDNF*, *HPP1*, and *MYOD1* correlated with the inflammatory status in 28 surgical specimens from medication-resistant patients undergoing colectomy. In line with previous reports, a significant correlation between the methylation of *SLIT2* and *TGFB2* promoters and endoscopic and histological acute inflammation has been shown[38-42]. In our cohort of mostly asymptomatic patients, microscopic inflammation was present in more than 50% of patients analyzed with endoscopic remission (ER). Microscopic inflammation may be used as a refined surrogate of disease activity in the absence of endoscopic activity[38-43] and might eventually be designated clinically relevant to dysplasia assessment because microscopic colitis has been associated with CAC[11].

In UC patients, low grade dysplasia[29,30] and neoplasia have been reported to be more frequent in the rectosigmoid colon[28]. It has been proposed that this might be secondary to a higher degree of inflammation. Of note, the methylation of *SLIT2* and *TGFB2* is more frequent in the rectosigmoid colon, suggesting an association between methylation, inflammation and dysplasia. However, in our study, both inflammation and location were independently associated with methylation; thus, this relationship may be more complex. Further studies will be needed to clarify this relation and to explore whether controlling inflammation reduces the risk of dysplasia.

Novel biomarkers should be introduced in routine clinical settings if they add information to those currently used. In spite of the evident correlation between histological inflammation and methylation, neither disease duration nor extension, the most commonly used high risk-criteria, was correlated with either histological activity or methylation. These two parameters may be eventually incorporated as risk factors or even as surrogate endpoints for treatment efficacy. This hypothesis

is further supported by the high prevalence of *SLIT2* among "endoscopically healed" patients.

The iterative assessment of the inflammation and methylation status has shed further light onto this correlation. On the one hand, the lack of methylation persisted when histological healing was maintained (the most prevalent situation in our cohort), suggesting a protective effect against aberrant DNA methylation. On the other hand, up to one-third of the cases analyzed (24 of 66) changed their methylation status (gain or loss indistinctly) during follow-up. Methylation tended to accumulate with inflammation, as reported in Barrett's esophagus[23], chronic biliary tract inflammation[22] and intestinal cancer mouse models[44,45]. Moreover, the reversibility of methylation status has been previously documented in gastric carcinogenesis after *H. pylori* eradication[21].

Our study has several limitations. First, due to the limited sample size, the association between inflammation and the methylation of these genes should be validated, in particular those observed in the longitudinal study. Second, because the study was not designed to assess the reversibility of methylation when healing the microscopic inflammation, further studies are needed to confirm this hypothesis due to its potential therapeutic implications. If the reversibility of methylation after the mucosa has healed could be demonstrated, it would be reasonable to propose histological healing as an endpoint of the treatment. Finally, we chose 2 genes that had previously demonstrated distinct patterns in patients at increased risk and low risks of developing dysplasia or cancer at our center. However, many other genes have been studied in this field, and therefore, in the future, a more extensive study could include a panel with more genes.

In conclusion, this study showed that methylation status is an accurate predictor of microscopic inflammation in chronic UC or CC patients. Microscopic inflammation was frequent among patients with no endoscopic activity. We also demonstrated that a distal location (the rectosigmoid colon), together with microscopic inflammation, constituted independent risk factors for methylation. Finally, in our population, we observed that the maintenance of ER was protective against de novo methylation, although further longitudinal studies are needed to confirm this.

COMMENTS

Background

Methylation status has been proposed as one of the pathways of colitis-associated cancer in ulcerative colitis (UC) and Crohn's colitis (CC) patients. However, there are scarce data about its relationship with endoscopic and histological inflammation.

Research frontiers

The carcinogenesis process of colorectal cancer in inflammatory bowel diseases is multifactorial, and more studies are necessary to improve understanding of this topic.

Innovations and breakthroughs

Methylation status correlated better with histological than with endoscopic activity, which may indicate the importance of controlling microscopic inflammation in this group of patients.

Applications

A better understanding of the relationships among the endoscopic, histological and molecular changes occurring in inflammatory bowel disease patients may help to optimize the management of this disease.

Peer review

This is a study to further elucidate the clinical role of two methylation markers in high risk patients with UC and CC. Despite limited by sample size, the study provides useful data that consolidate the pathological role of these markers.

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