IL-8 and LYPD8 expression levels are associated with the inflammatory response in the colon of patients with ulcerative colitis

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Abstract. Ulcerative colitis (UC) is an idiopathic chronic inflammatory disorder affecting the large intestine, which may involve mucosal degeneration. Glycoproteins, mucin2 (MUC2) and the LY6/PLAUR domain containing 8 (LYPD8) are present on the mucous layer of the colon and can hinder the invasion of bacteria, thus contributing to the prevention of colitis. The present study investigated the expression levels of interleukin-8 (IL-8), MUC2 and LYPD8 on the mucous membranes of patients with UC. A total of 18 patients with UC (6 females and 12 males) were examined. Biopsies of the lesions as well as matching normal membranes were obtained and the mRNA expression levels of IL-8, MUC2 and LYPD8 were compared. LYPD8 expression was downregulated in the lesions and the relapsing-remitting subtype of lesions was associated with higher levels of MUC2 expression compared with single attack and chronic lesions subtypes. A positive correlation between Matts' histopathological grade and IL-8, as well as a negative correlation between Matts' histopathological grade and LYPD8 were observed. The expression levels of LYPD8 were lower in highly active lesions and these levels decreased according to the intensity of the mucosal inflammation. Conversely, an increase in MUC2 expression levels may reflect the recovery of the outer mucus layer in the remission phase. Therefore, the examination of MUC2 and LYPD8 expression levels may be useful indicators of mucosal healing in patients with UC.

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Abbreviations: UC, ulcerative colitis; MUC2, mucin2; LYPD8, LY6/PLAUR domain containing 8; IL-8, interleukin-8

Key words: ulcerative colitis, MUC2, LYPD8, IL-8, mucosal healing

Introduction

Ulcerative colitis (UC) is a chronic inflammatory disease of the colon characterized by recurrent periods of clinical remission and disease relapse (1). The incidence of colorectal cancer is increasing, and UC is a risk factor of colorectal cancer (2,3). Despite advances in therapeutic options, in a cohort study conducted in New Zealand between 2005 and 2015, 2.7% of all patients with UC went onto develop colorectal cancer (4). UC has a marked negative impact on the quality of life of the patients. The onset and reactivation of UC is associated with genetic abnormalities that cause defects in the mucosal barrier and alters the balance between the beneficial and pathogenic enteric bacteria (5). In the colon, the mucus layer is primarily composed of a large gel-forming mucin containing the glycoprotein mucin2 (MUC2) (6). MUC2 serves an important role in protecting the colon against colitis, and this has been demonstrated by the fact that MUC2-deficient mice develop spontaneous colitis (6,7).

Previously, Okumura *et al* (8) identified a novel protein present in the inner mucosal layer called LY6/PLAUR domain containing 8 (LYPD8) protein, which is selectively expressed in epithelial cells at the uppermost layer of the large intestinal gland. The group demonstrated that LYPD8 can bind to the flagellae (composed of polymerized flagellin proteins) of live bacteria. LYPD8-/- mice possess slightly increased numbers of *Proteus* species in the luminal regions of the colon compared with wild-type mice (8). *Proteus* has been associated with the pathogenesis of inflammatory bowel diseases in both mice and humans (9,10) and LYPD8 promotes the segregation of flagellated bacteria and colonic epithelia, thus reducing the risk of intestinal inflammation (8-11).

As mentioned above, there are several reports on the role of MUC2 and LYPD8 in UC; however, only two studies have examined the role of MUC2 and LYPD8 in the context of severity of inflammation and gene expression in UC (12,13). Furthermore, to the best of our knowledge, there are no studies comparing their gene expression in the lesioned and non-lesioned regions of the colon in patients with UC. Therefore, the present study aimed to investigate the association between the severity of inflammation and MUC2 and LYPD8 expression levels in these regions.

Patients and methods

Patients. Patients with UC who underwent treatment at Tottori University Hospital (Tottori, Japan) and Nagasaki University Hospital (Nagasaki, Japan) between August 2018 and July 2019 were enrolled. Patients who disagreed to participation in the study were excluded. A total of 18 patients with UC in the acute and remission phases, including 6 females and 12 males, were examined. The mean age ± standard deviation was 41.1±14.7 years (range, 18-74 years). UC was diagnosed based on clinical symptoms, the results of endoscopy, X-rays and histological findings. Patients with UC were treated with 5-aminosalicylic acid, prednisolone (PSL), granulocyte apheresis (G-CAP) and azathioprine (AZA). Biopsies of the lesioned and non-lesioned areas of the colon were collected from the same patient for 9-342 months following the initiation of treatment. The distinction of normal or lesioned regions was based on endoscopy images, and the expression levels of IL-8, MUC2 and LYPD8 were compared between the lesioned and non-lesioned areas.

Samples were stratified into three groups based on the Matts' histopathological grade (14); grade 1 (n=20), grade 2 (n=9) and grade 3 (n=7); for all regions, and the expression levels of IL-8, MUC2 and LYPD8 in the different grades were compared. All cases were anonymized prior to analysis and written informed consent was provided by all patients. The present study was approved by The Institutional Review Board of Tottori University (Tottori, Japan) and was performed in accordance with the Declaration of Helsinki (15).

RNA extraction. The total RNA, including mRNA and miRNA, from the tissues was extracted from biopsies using an miRNeasy Mini kit (Qiagen China Co., Ltd.). The RNA was quantified using a BioSpec Nano Spectrophotometer (Shimadzu Corporation) and the extracted RNA samples were stored at -80˚C until further use.

Reverse transcription-quantitative (RT-q)PCR. RNA was reverse transcribed into cDNA using a High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.). The reverse transcription reactions were performed in aliquots containing 1 μg total RNA, 1X RT buffer, 4 mM dNTP mix, 1X RT random primer, 50 units Multiscribe reverse transcriptase, 20 units RNase inhibitor and nuclease-free water added to a final volume of 20 μl. The RT temperature protocol was: 25˚C for 10 min, 37˚C for 120 min and 85˚C for 5 min. The primer sequences for qPCR were as follows: IL-8 forward, 5'-TTTTGC CAAGGAGTGCTAAAGA-3' and reverse: 5'-AACCCTCTG CACCCAGTTTTC-3'; MUC2 forward, 5'-ACAACTACTCCT CTACCTCCA-3' and reverse, 5'-GTTGATCTCGTAGTTGAG GCA-3'; LYPD8 forward, 5'-CTGAAGAACGTGTCCAGC AA-3' and reverse, 5'-CTGACAGGTGGCGTTACTGA-3'; and β-actin forward, 5'-GCATCCTCACCCTGAAGTA-3' and reverse, 5'-TGTGGTGCCAGATTTTCTCC-3'. qPCR was performed in 20 μl aliquots containing 1 μl RT products using a 4 μl LightCycler® FastStart DNA Master PLUS SYBR Green I (cat. no. 03515869001; Roche Diagnostics), 0.5 μM of each primer and 14.6 μl nuclease-free water on a Real Time PCR LightCycler 1.5 Complete system (Roche Diagnostics). The thermocycling conditions were as follows: Initial denaturation step at 95˚C for 10 min, followed by 45 cycles of 95˚C for 10 sec, 60˚C for 10 sec and 72˚C for 10 sec. The quantification cycle threshold (Cq) was recorded for each mRNA using LightCycler version 3.5.28 (Roche Diagnostics) and β-actin was used as the endogenous control for data normalization. The relative expression was calculated using the following formula $2^{-\Delta\Delta Cq} = 2^{-(\Delta Cq, \text{reagent treatment}-\Delta Cq, \text{control})}$ (16).

Statistical analysis. Differences in sex, presence/absence of recurrence, presence/absence of treatments for each drug, and lesioned/normal regions, were analyzed using a Student's t-test. Extent of disease, disease type and the presence/absence of treatments for each drug were analyzed using a one way ANOVA with a Tukey's post-hoc test. The correlation between Matts' histopathological grade and gene expression was analyzed using Kendall's Tau rank correlation coefficient. Data are expressed as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patients. Of the 18 patients, 11 had pancolitis, 3 had distal UC and 4 had left-sided UC. There were 8 cases of single attack only, 6 cases of relapsing-remitting sub-type and 4 cases of chronic continuous sub-type UC. Regarding treatment of these patients: 10 patients were treated with 5-ASA; 3 patients were treated with 5-ASA and PSL; 1 patient was treated with 5-ASA and G-CAP; 1 patient was treated with 5-ASA and AZA; 1 patient was treated with PSL and AZA; 1 patient was treated with 5-ASA, PSL and AZA; and 1 patient was treated with 5-ASA, PSL, G-CAP and AZA (Table I).

Background. Men had significantly higher mRNA expression levels of MUC2 expression compared with females (0.115±0.240 vs. 0.0653±0.0376, respectively; $P<0.05$; Table II). In addition, patients with the relapsing-remitting sub-type displayed higher levels of MUC2 expression compared with patients with the single attack only sub-type and the chronic continuous sub-type (0.355±0.266 vs. 0.0711±0.0612, respectively; $P<0.01$ and 0.355±0.266 vs. 0.0495±0.0320, respectively; both $P<0.01$; Table II). No association was observed between IL-8, LYPD8 and MUC2 expression levels and the different treatments (data not shown).

Normal vs. lesion. The expression levels of IL-8 were slightly higher in the lesioned regions compared with the normal regions of the colon (0.000431±0.00445 vs. 0.000327±0.000601, respectively; $P<0.05$; Fig. 1). In contrast, the decrease in LYPD8 expression levels in the lesioned regions compared with normal regions was significant (0.0156±0.0335 vs. 0.0591±0.0485, respectively, $P<0.001$; Fig. 1).

Matts' histopathological grade. There was a positive correlation between Matts' histopathological grade and IL-8 expression levels ($T=0.256$; $P<0.01$; Fig. 2), whereas a negative correlation was observed between Matts' histopathological grade and LYPD8 expression levels ($T=-0.411$; $P<0.01$; Fig. 2). There was no correlation between Matts' histopathological grade and MUC2 expression levels (Fig. 2).

Table I. Clinical characteristics of 18 patients with ulcerative colitis.

Characteristics	n
Age, years, mean (SD)	41.1 (14.7)
Sex	
Male	12
Female	6
Location of disease, n (%)	
Pancolitis	11 (61.1)
Left-side UC	4 (22.2)
Distal UC	3 (16.7)
Disease type, n (%)	
Single attack only	8 (44.4)
Relapsing-remitting	6 (33.3)
Chronic continuous	4 (22.2)
Acute fulminant	0 (0)
Treatment, n (%)	
5-ASA	10 (55.5)
5-ASA, PSL	3 (16.7)
5-ASA, G-CAP	1 (5.6)
5-ASA, AZA	1 (5.6)
AZA, PSL	1 (5.6)
5-ASA, PSL, AZA	1 (5.6)
5-ASA, PSL, G-CAP, AZA	1 (5.6)

5-ASA, 5-aminosalicylic acid; PSL, prednisolone; G-CAP, granulocyte apheresis; AZA, azathioprine; SD, standard deviation.

Table II. Gene expression of IL-8, MUC2 and LYPD8 classified by sex, location of disease, disease type and presence of recurrence in the lesioned colon of patients with ulcerative colitis.

Characteristics	IL-8, $x10^{-4}$	MUC2, 10^{-2}	Lypd8, $x10^{-2}$
Sex			
Male	4.07±38.3	11.5±24.0[a]	3.65±4.24
Female	2.37±7.26	6.53±3.76	4.37±5.76
Location			
Pancolitis	3.05±21.0	8.99±23.7	4.23±4.51
Left	16.2±58.5	5.92±17.8	3.18±6.54
Distal	3.31±5.26	3.02±15.5	1.87±1.84
Sub-type			
Single attack only	3.15±5.91	7.11±6.12	4.37±4.88
Relapsing	11.2±28.5	35.5±26.6[b,c]	3.47±2.75
Chronic	4.83±60.5	4.95±3.20	5.26±6.49
Recurrent	16.5±62.7	21.6±20.4	1.60±4.86
Non recurrent	3.24±10.1	7.00±21.0	4.01±4.80

[a]P<0.05, [b]P<0.01 vs. single attack only sub-type; [c]P<0.01 vs. chronic sub-type. Data are presented as mean ± standard deviation. IL-8, interleukin-8; MUC2, mucin2; LYPD8, LY6/PLAUR domain containing 8.

Discussion

While studying the relationship between LYPD8 expression levels and UC, Okumura *et al* (8) reported that LYPD8 expression levels were decreased in patients with UC, indicating that LYPD8 may function in the pathogenesis of inflammatory bowel diseases. The present study showed that LYPD8 expression levels were lower in the lesioned compared with matching non-lesioned regions of the colon in patients with UC. This suggests that the expression levels of LYPD8 are specifically downregulated in the inflamed mucosa of patients with UC and that LYPD8 may be a novel inflammatory marker. Furthermore, the present study showed that LYPD8 expression levels and pathological mucosal inflammation were associated. LYPD8 is expressed in the uppermost layer of the colonic gland, and is secreted by the epithelial cells (8). Destruction of the mucosal layer and depletion of mucin results in the downregulation of LYPD8 expression levels in patients with UC (8). The present study also showed that decreased expression levels of LYPD8 were associated with the severity of mucosal inflammation. Furthermore, LYPD8 expression was not affected by sex, location of lesions, type of treatment, combination of treatments, and presence/absence of recurrence; therefore, it specifically reflected mucosal inflammation.

IL-8 expression levels are high in the inflamed mucosa of patients with UC (17-19) and Pearl *et al* (12) reported that there was an association between the severity of inflammation and the mucosal concentration of IL-8. The present study showed that IL-8 expression levels were upregulated in the inflamed regions of the colon in patients with UC, and this effect was independent of patient background factors such as sex, location of lesions, type of disease, combinations of treatment, and presence/absence of recurrence. Mazzucchelli *et al* (20) reported that IL-8 expression is restricted to histopathologically inflamed regions of the colon. Therefore, IL-8 expression may not reflect patient background, but instead only the severity of inflammation.

The intestinal tracts of mice infected with the apicomplexan parasite *Eimeria papillate* showed greater MUC2 expression levels in females compared with males (21); however, to the best of our knowledge, no previous studies have reported the association between MUC2 levels with sex differences in humans with UC. There may be differences in the sensitivity of epithelial cells to UC due to sex, resulting in differences in MUC2 expression. MUC2 expression levels do not correlate with the severity of inflammation (6,22). The present study showed that MUC2 expression levels were higher in patients who had undergone a greater number of cycles of relapse and remission, and this suggests that increased MUC2 expression levels may reflect the number of courses of relapse and remission of mucosal inflammation in UC. To the best of our knowledge, there are no studies focused on MUC2 expression in patients examining the location of the lesion, combinations of treatment, and presence/absence of recurrence; thus, further studies are required.

Previous studies have revealed that mucosal healing is an important therapeutic goal for successfully treating patients with UC as it may improve patient outcomes, potentially changing the course of the disease by inducing sustained

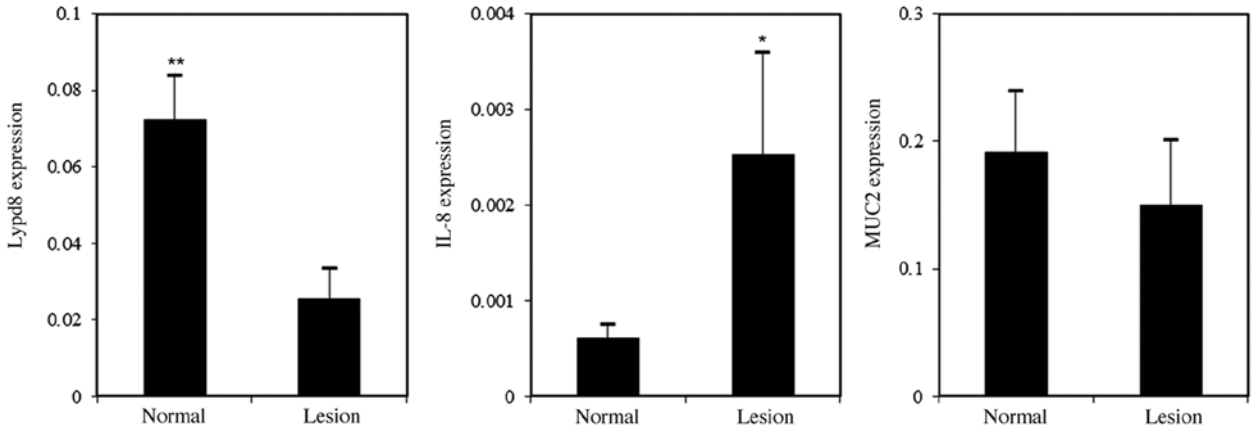

Figure 1. Comparison of LYPD8, IL-8 and MUC2 expression levels in normal vs. lesioned colon tissues in patients with UC. n=18. *P<0.05, **P<0.01. LYPD8, LY6/PLAUR domain containing 8; IL-8, interleukin-8; MUC2, mucin2.

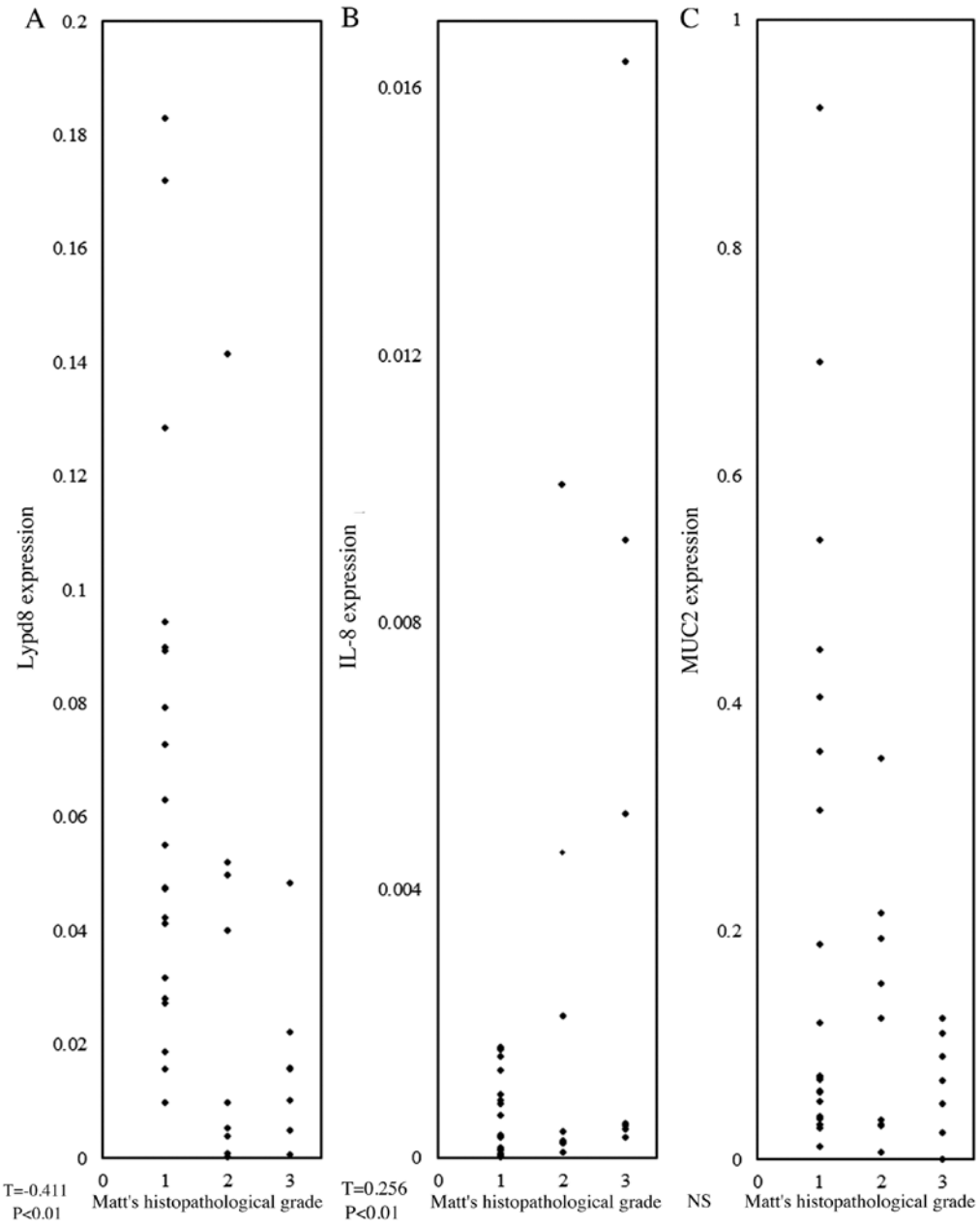

Figure 2. Correlation between Matts' histopathological grade and the expression levels of (A) LYPD8, (B) IL-8 and (C) MUC2. n=36. LYPD8, LY6/PLAUR domain containing 8; IL-8, interleukin-8; MUC2, mucin2; NS, not significant; T, tau.

clinical remission and reducing the need for hospitalization and surgery (23-25). However, as there is no established definition of mucosal healing, and it is difficult to assess the efficacy of various drugs (24). One of the most commonly used definitions of mucosal healing, as stated by the International Organization of Inflammatory Bowel Disease, is the 'absence of friability, blood, erosions, and ulcers in all visualized segments' corresponding to a Mayo Endoscopic sub score between 0 and 1 (26). However, this definition does not describe the histological healing of the mucosa (27). Additionally, although discrepancies between histological and endoscopic data exist, these are rarely assessed in therapeutic trials (28). Mucosal healing may be multilaterally assessed by combining the expression of mucosal markers such as LYPD8 with endoscopic and histological indicators.

In conclusion, it was shown that IL-8 and LYPD8 were specifically expressed in the inflamed mucosa compared with non-inflamed mucosa of patients with UC, and that expression was correlated with the severity of inflammation. Using IL-8 and LYPD8 expression as indicators, it may be possible to more accurately determine the outcomes of therapeutic treatments in patients with UC.

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Availability of data and materials

All data generated or analyzed during the present study are included in this article.

Authors' contributions

TO and TK made substantial contributions to the acquisition, analysis and interpretation of the data, as well as in drafting the manuscript. TK also established the LYPD8 primers. KH and KN helped in acquiring the data and performed the biopsies. YI and NU conceived and designed the study. HI assisted in the conception and design of the study as well as in drafting the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by The Institutional Review Board of Tottori University (Tottori, Japan) and was performed in accordance with the Declaration of Helsinki. Written informed consent was provided by all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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