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Original Article

Promotor Hypomethylation of *TFF1* Gene in Ulcerative Colitis is Positively Correlated with Aging

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Abstract

Background: Epigenetic modifications exhibit promising evidence in the etiology and prognosis of important diseases such as inflammatory bowel diseases (IBD). In addition to complex factors involved in IBD, a trend toward better prognosis has been reported in older ages of disease onset, specifically in ulcerative colitis (UC). The gastrointestinal mucous layer is one of the important components that is disturbed in the disease course. The integrity of this layer is maintained with an anti-inflammatory factor called trefoil factors (TFF). We investigated the methylation status of the *TFF1* gene in UC patients alongside with correlation of its alteration level with age of disease onset.

Method: We analyzed the promoter methylation status of the *TFF1* gene, using the real-time quantitative multiplex methylation specific PCR (QM-MSP). DNA was extracted from colorectal biopsies of 15 ulcerative colitis and 14 healthy controls. Correlation analysis was performed between unmethylated DNA level and age through the Pearson correlation coefficient (PPC) test and simple linear regression models.

Results: Our data provided a significant positive correlation between age and TFF1 hypomethylation in ulcerative colitis patients. However, no significant difference was observed in overall TFF1 methylation status between ulcerative colitis patients and control subjects.

Conclusion: This finding suggests the association between epigenetic upregulation of the *TFF1* gene with disease mildness in older patients.

Keywords: Inflammatory Bowel Disease; Ulcerative Colitis; Trefoil Factor; Epigenetics; Methylation; Aging

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Introduction

Inflammatory bowel diseases (IBD), comprising Crohn's Disease (CD) and Ulcerative Colitis (UC), are a group of chronic inflammatory diseases that significantly affect the patient's quality of life. UC and CD differ on the site and extension of the inflammation. CD can occur from mouth to anus and cause severe inflammation which may extend to the entire thickness of the bowel wall. However, UC is usually restricted to the innermost layer of the colon and rectum. Although the exact mechanisms underlying the occurrence and prognosis of both diseases demand further studies, several factors are reported to be involved such as genetic, immune responses, intestinal microbiota, and interaction of genome with environmental factors without alteration in genome sequence, regarded as "epigenetic" factors (1-3).

Epigenetic modifications of promoter can occur through cytosine methylation in specific cytosine guanine-rich sites called CpG islands. Several genes may undergo aberrant methylation in promoter sites such as hypo- or hypermethylation which may lead to gene over- or under-expression, respectively (2). Aging may also alter the methylation state of specific genes through various mechanisms (4). On the other hand, the age of the IBD onset has been reported to influence disease severity and prognosis. For instance, pediatric UC patients usually experience severe and extensive inflammation which is contrary to the usual stable trend of UC in elderly adults. However, severity of CD is much less dependent on age (5, 6). Therefore, studying underlying mechanisms for this age-dependent UC prognosis is of interest. Among various factors perturbed in this disease, the mucin layer and its associated factors seem intriguing in disease propagation and prognosis. The Mucin layer provides a protective barrier between the epithelial layer and gut microbiota (7). Mucin is co-secreted with small peptides called trefoil factors (TFF) which participate in mucosa protection and repair through increasing mucin layer viscosity and elasticity. Therefore, these factors are considered to exert their protective role during the course of gut inflammatory diseases such as IBD (8). Trefoil factors (TFF) are basically expressed in gastrointestinal tract epithelial cells and their genes are clustered on chromosome 21q22.3. They play a role in epithelial defense and repair (9, 10). There are 3 subtypes of trefoil factor family (TFF) among which TFF1 seems more intriguing to be observed in IBD. Normally, TFF1 is expressed in foveolar cells of superficial epithelium in the stomach and maintains its normal structure and functions (11, 12). Clinical studies on IBD demonstrated that gene expression of all three TFFs was altered at the site of mucosal damage in IBD (13, 14). This alteration is reported to be the response of epithelial cells to inflammatory cytokines, as their innate protection. Due to the anti-inflammatory roles of TFF1, it's considered a protective and curative factor in IBD patients, specifically in UC patients (15). Variations in the expression of TFF can be associated with diverse environmental factors acting through Epigenetics. For instance, TFF1 promoter methylation variations have been reported in retinoblastoma cell lines and prostate cancer (16, 17). We hypothesized that epigenetic control of TFF expression through methylation of CpG Islands may have a role in UC patients according to their age, as it plays an important role in UC prognosis and severity. Therefore, a correlation analysis between TFF methylation and aging in UC is reported.

Methods

Tissue samples

Colorectal samples were obtained from men and women undergoing colonoscopic mucosal resection at the gastroenterology clinics of Kasra and Laleh hospitals in Tehran, Iran. The study population consisted of 15 UCs (8 females, 7 males), and 14 age-matched healthy controls (8 females, 6 males) undergoing routine colonoscopy checkups. Healthy controls didn't have any history of IBD or other gastrointestinal abnormalities, approved by histopathological examination. All patients were informed of the use of their specimen and written consent was obtained from all participants. The tissue collection and protocol of this study were approved by the Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran.

DNA preparation and bisulfite conversion

A high Pure PCR Template Preparation Kit (Roche) was applied to isolate the genomic DNA of the subjects' colonic mucosa. Bisulfite mod-

ification of genomic DNA was done by MethylEdge™ Bisulfite Conversion System (Promega, Madison, WI) through treatment with sodium bisulfite. During the conversion process, all cytosine residues are converted to uracil whereas 5-methylcytosine (5mC) remains unmodified. Bisulfite-modified DNA specimens were aliquoted and stored at -20°C **(Figure 1)**.

Methylation Analysis

Real-time quantitative multiplex methylation-specific PCR (QM-MSP) based on SYBR green was applied to evaluate the methylation status of CpG sites across the promoter region of the *TFF1* gene [18]. QM-MSP required two sequential steps of PCR reactions performed with a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). The first pre-amplification PCR reaction (the multiplex step) was carried out with MethySYBR primers including external forward primer (EXT-F; 5'-AGTTTAGGTTTAGACGGAATGG-3') and external reverse primer (EXT-R; 5'-TCTCCTCCAACCTAACCTTAAT-3'). The primer set was designed to enable the simultaneous amplification of many discrete target alleles in a single reaction. The PCR reaction was performed in a volume of 25 µl containing 1 µl of converted genomic DNA following this protocol:

1. Denaturation at 95°C for 5 min
2. 30 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s
3. Final extension at 72°C for 5 min

In the second round of PCR, the specific methylated target is quantified from multiplex step products using both nested methylation-independent and methylation-specific primer sets including nested methylation-specific forward (FM; CGGAATGGGTTTTATGAGTT-3') and reverse primer (RM; 5'- CCGCCAAAATAAATACTATACTCA-3') (19). The bisulfite-treated DNA was PCR amplified in a 10 µl reaction volume containing 5 µl SYBR® Green Master Mix, 0.25 µl of each of the methylated primers, 3.5 µl DDW,

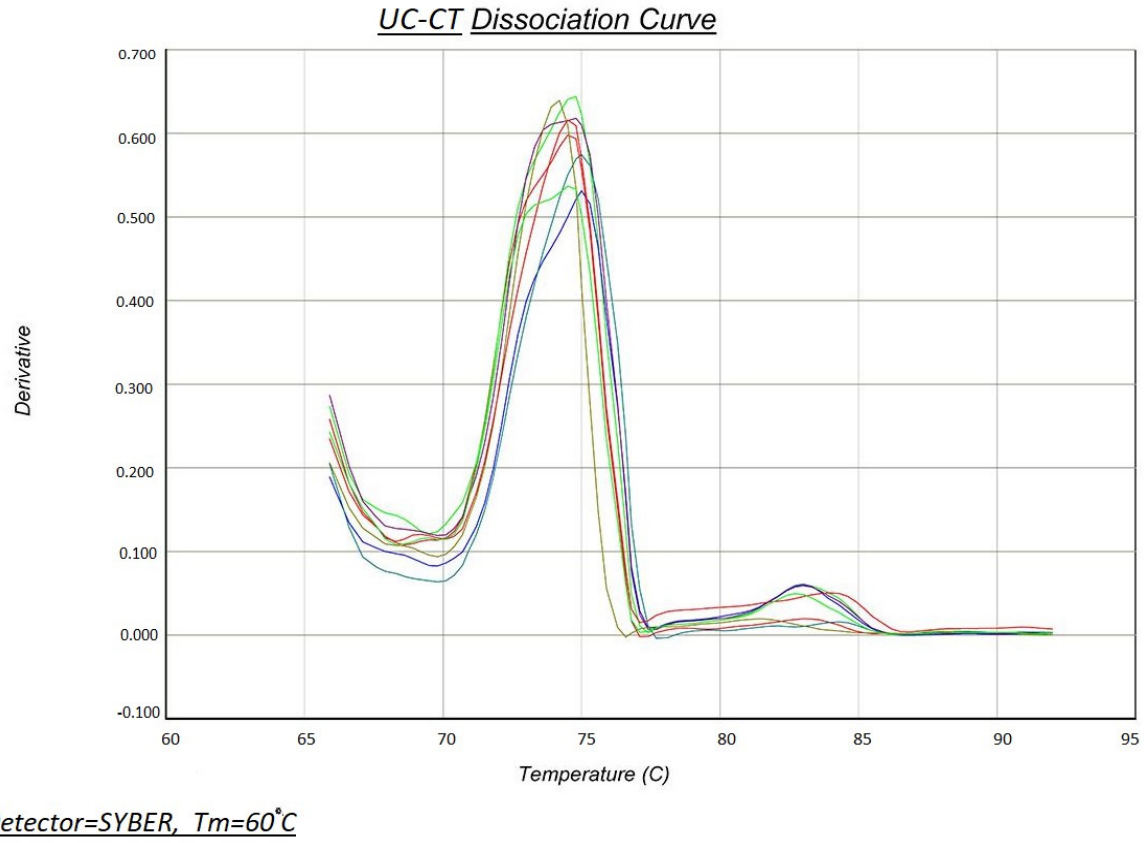

Figure 1. After bisulfite conversion, methylated DNA (with cytosine) exhibits higher melting temperature than unmethylated DNA (containing thymine). As seen in UC patients and CT (controls), the higher peak of the melting curve is allocated to unmethylated DNA.

and 1 µl of bisulfite-treated DNA. Cycling conditions were:

1. 1 min at 95°C
2. 30 cycles of 30 s at 94°C
3. 1 min at 60°C
4. 30 s at 72°C subsequently followed by 5 min at 72°C

CpG island prediction and primer blasting were performed using the MethBlast tool. The methylation profile of the promoter CpG islands was defined based on the UCSC database. No untreated template controls were included in each run as negative controls. Fully converted methylated human plasmid DNA was used as a positive control for MSP in each run to serve as the 100% methylated reference for calculating the relative methylation percentages of DNA samples.

The ΔΔCq method was used to calculate the ratio of unmethylated versus total amplifiable bisulfite-treated DNA. The cycle of quantification (Cq) for the reaction between methylation-specific primers (MSP) and bisulfite-specific primers (BSP) was also obtained (20, 21). Using a reference sample for standardization indicates the relative difference between the template of interest and a control template:

$\Delta\Delta Cq = \Delta Cq_{\text{sample}} - \Delta Cq_{\text{plasmid}}$

$\Delta Cq_{\text{sample}} = Cq_{\text{MCP}} - Cq_{\text{BSP}}$

$\Delta Cq_{\text{plasmid}} = Cq_{\text{MCP}} - Cq_{\text{BSP}}$

All cycle threshold (CT) values were obtained in the exponential phase and normalized by subtraction of the CT value. After normalization with the expression of PCR products amplified by external nested primers as internal control (BSP), the fold change in target gene samples was calculated using the $2^{-\Delta\Delta CT}$ method:

$\Delta CT = CT_{\text{target gene}} - CT_{\text{BSP products}}$

$\Delta\Delta CT = \Delta CT_{\text{(samples)}} - \Delta CT_{\text{(controls)}}$

Unmethylated DNA level= $2^{-\Delta\Delta CT}$

Statistical analysis

Unmethylated DNA levels were compared between the three study groups using a one-way analysis of variance. The Pearson correlation coefficient (PPC) test was used to assess the correlation between age and hypomethylation. Furthermore, simple linear regression models were constructed for the prediction of unmethylated DNA levels based on age. Based on these models, measurement outliers were determined as data points with a significantly extreme studentized residual value at the level of alpha = 0.05. Thus, two and one patients were identified as outliers in the UC study groups, respectively **(Figure 2)**. All statistical analysis was performed using R version 3.5.1.

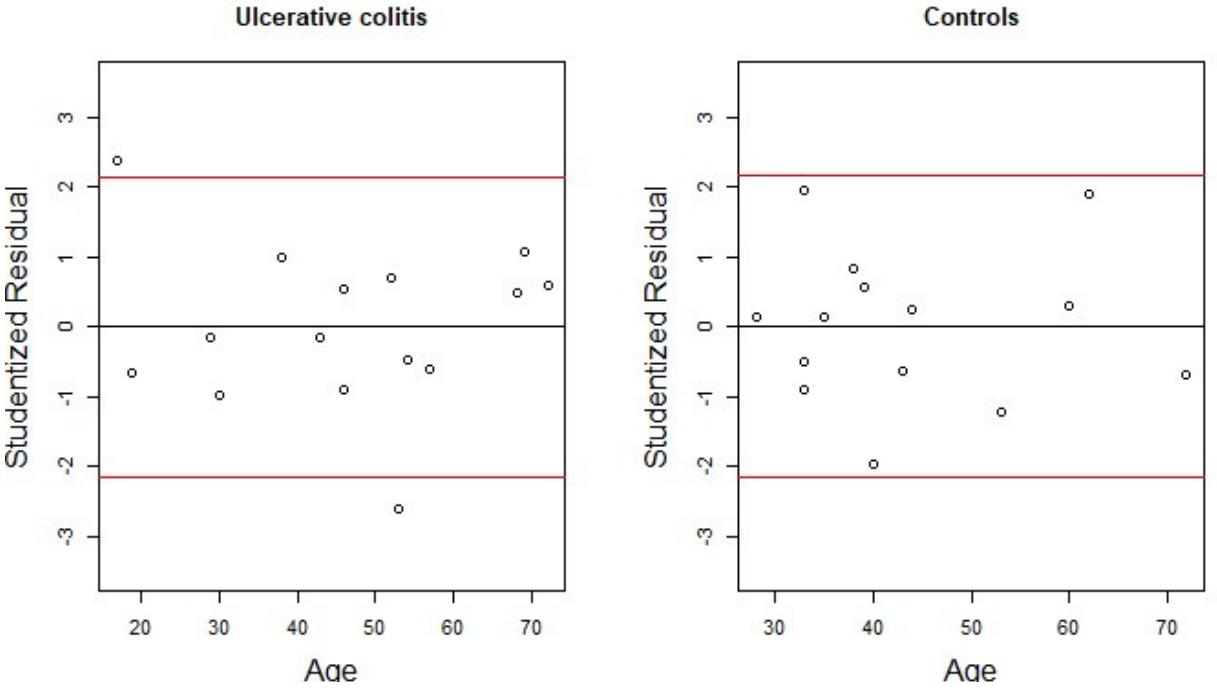

Figure 2. The extreme lines show the statistical significance level at alpha = 0.05 (±2.145 for UC [df = 14]; ±2.160 for Controls [df = 13]).

Results

Methylation state of the *TFF1* gene in UC patients

DNA methylation in the TFF1 promoter was assessed in 15 UC and 14 healthy controls. T-test did not show a significant difference in hypomethylation levels between the two study groups, *P=0.973,* 95% CI:(-0.3069 - 0.2972) **(Figure 3)**. This suggests an almost identical methylation state of the TFF1 promoter in UC patients and the control group in general.

Correlation of TFF1 promoter unmethylation and age in UC

We also studied the relationship between age and unmethylated DNA levels in patients. Pearson correlation coefficients were calculated both including and excluding outliers and are presented in **Table 1**. A significant correlation is observed in UC patients (r=0.712, *P=0.006)*. No significant correlation was observed in controls which strengthens our hypothesis on IBD-associated alteration on promoter methylation state while aging, not sole aging-associated epigenetic regulations. Furthermore, simple linear regression models were constructed to predict hypomethylation levels based on age. A significant regression equation was found for patients suffering from ulcerative colitis (F(1, 11)= 11.33, *P= 0.006*), with an R2 of 0.507 after excluding the outliers. A UC patient's predicted unmethylated DNA level is equal to 0.427 + .016 (age) when age is measured in years. However, there does not seem to be any relationship between hypomethylation and age in healthy individuals **(Figure 4)**. Therefore, age seems to be a valid predictor of hypomethylation in UC patients.

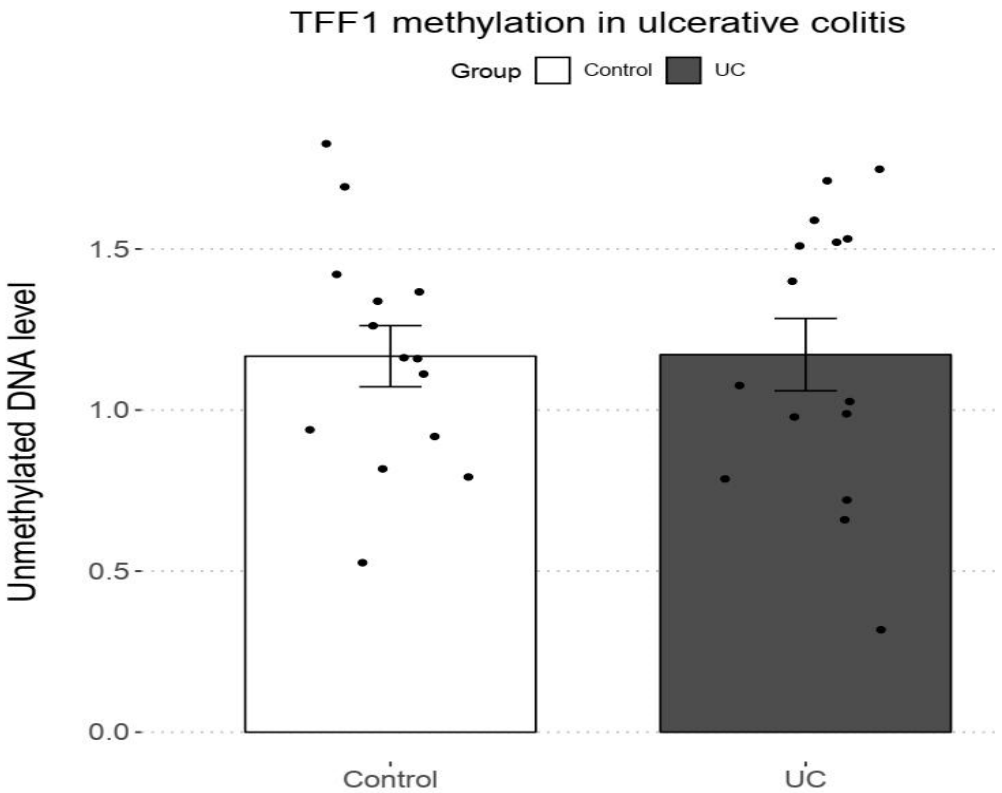

Figure 3. The means and error bars are shown along with data points

Table 1. Correlation between age and hypomethylation

	n	Including outliers		Excluding outliers	
		r	*P*-value	r	*P*-value
Ulcerative colitis	15	.291	.293	.712	.006

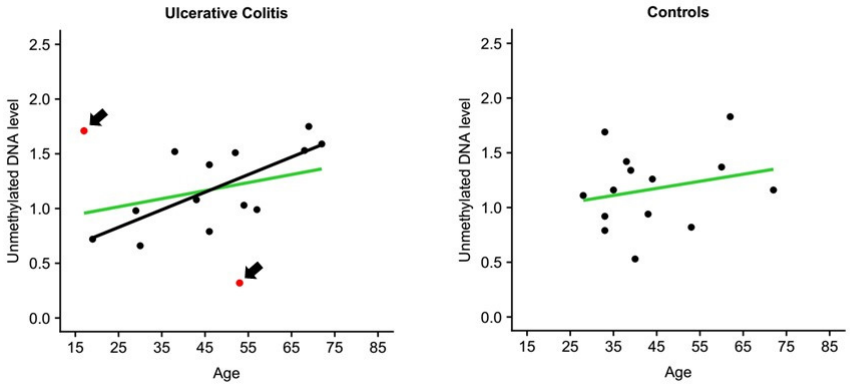

Figure 4. The arrows show outlier data points. Simple linear regression models are shown both including (in) and excluding (ex) outliers. The formulas for the lines are as follows: UC: (in) .832 + .007 (age), R2= .085, F(1,13)= 1.203, *P*= .293; (ex) .427 + .016

Discussion

Our results demonstrated a promising correlation between age and TFF1 hypomethylation in UC patients. It's been reported that the presentation of IBD becomes prominently different as the age of the disease onset increases. Older patients tend to have milder and more stable disease. However, initial lesions of young adults and pediatrics are prone to further extension. Especially in UC patients, our results were consistent with the previous reports that the stability of UC lesions is reported in most elderly patients. Relapses are also more common in young patients in comparison with older ones (5, 22). Therefore, positive regression in TFF1 hypomethylation and age in UC patients rationalizes its protective role in alleviating disease course in old ages (23). This alteration in promoter methylation may be due to the endogenous epigenetic tools of cells or the accumulative effects of lifestyle factors like smoking, breathing toxins, drugs, etc. (4). Our data didn't show any significant difference between promoter methylation of TFF1 in UC patients in comparison with healthy controls, despite reports on newly upregulated TFF1 levels in these patients (24). This discrepancy may be due to the mild disease state in our 15 cases of UC or other mechanisms of TFF1 regulation of gene expression in these patients. DNA methyl transferases (DNMT) play an important role in methylation of specific gene promoters which provides a mechanistic hypothesis for our results in the TFF1 methylation state. DNMT1 activity is reportedly upregulated in UC, which may explain insignificant hypomethylation of the TFF1 promoter, and it may also indicate that the baseline genome methylation variations should be considered in the analysis (25). The mechanisms involved in this protective epigenetic regulation of TFF1 in elder patients are currently unknown but further studies on cytokine profile analysis in young and elderly patients may also reveal a mechanism for TFF1 hypomethylation in old age. There are reports that TNFα upregulates TFF1 in gastric cells (26, 27). Interestingly, factors involved in IBD initiation and propagation such as IL1β and IL6, are reported to downregulate TFF1 transcription (28, 29). In order to investigate the role of TFF1 downregulation in the disease initiation, further studies can analyze TFF1 promoter methylation levels before and after the disease incidence in the form of cohort studies. TFF1 has also demonstrated tumor-suppressing effects in several studies. For instance, TFF1 downregulation through promoter hypermethylation is reported in esophageal squamous cell carcinoma and gastric cancer (30-32). Therefore, many studies support the clinical application of TFF1 in IBD and even cancers; however, it still requires further studies to find the optimum dose and delivery methods (15, 33).

Conclusion

Our case-control study revealed that TFF1 methylation status is correlated with the age of ulcerative colitis patients. Due to the anti-inflammatory features of TFF1 and its increased promoter hypomethylation in elder patients, we suggest TFF1 hypomethylation as protective

feedback in severe IBD conditions. It could also be suggested that TFF1 methylation aberrations are a possible factor for patients' prognosis.

Funding

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Ethics

All patients were informed of the use of their specimen and written consent was obtained from all participants.

Authors' Contribution

SH, GS, and MS contributed equally to the study conception, conducting experiments, data analyses, and preparation of the manuscript. ND contributed to performing the experiments. NR supervised the study and contributed to the critical revision of the manuscript. All the authors read and approved the final manuscript.

Availability of data and materials

All data analyzed during this study are included in the figures of this published article; However, any additional data analyzed during the current study are available from the corresponding author if requested.

Conflict of interests

The authors declare that they have no competing interests

References

1. Ko JKAuyeung KK. Inflammatory bowel disease: etiology, pathogenesis and current therapy. Curr Pharm Des. 2014;20(7):1082-96.
2. Yi JMKim TO. Epigenetic alterations in inflammatory bowel disease and cancer. Intest Res. 2015;13(2):112-21.
3. Gajendran M, Loganathan P, Jimenez G, Catinella AP, Ng N, Umapathy C, et al. A comprehensive review and update on ulcerative colitis(). Dis Mon. 2019;65(12):100851.
4. Jung MPfeifer GP. Aging and DNA methylation. BMC Biol. 2015;13:7.
5. Duricova D, Burisch J, Jess T, Gower-Rousseau C, Lakatos PLEpiCom E. Age-related differences in presentation and course of inflammatory bowel disease: an update on the population-based literature. J Crohns Colitis. 2014;8(11):1351-61.
6. Roda G, Chien Ng S, Kotze PG, Argollo M, Panaccione R, Spinelli A, et al. Crohn's disease. Nat Rev Dis Primers. 2020;6(1):22.
7. Boltin D, Perets TT, Vilkin ANiv Y. Mucin function in inflammatory bowel disease: an update. J Clin Gastroenterol. 2013;47(2):106-11.
8. Thim L, Madsen FPoulsen SS. Effect of trefoil factors on the viscoelastic properties of mucus gels. Eur J Clin Invest. 2002;32(7):519-27.
9. Buda A, Jepson MAPignatelli M. Regulatory function of trefoil peptides (TFF) on intestinal cell junctional complexes. Cell Commun Adhes. 2012;19(5-6):63-8.
10. Emidio NB, Hoffmann W, Brierley SMMuttenthaler M. Trefoil factor family: Unresolved questions and clinical perspectives. Trends Biochem Sci 2019;44(5):387-90.
11. Song JY, Kim BW, Lee AW, Lee KY, Chung IS, Lee BI, et al. Expression of MUC5AC and Trefoil Peptide 1 (TFF1) in the Subtypes of Intestinal Metaplasia. Clin Endosc. 2012;45(2):151-4.
12. Cobler L, Mejias-Luque R, Garrido M, Pera M, Badia-Garrido Ede Bolos C. Activation of the NF-kB pathway downregulates TFF-1 in gastric carcinogenesis. Virchows Arch. 2013;463(4):497-507.
13. Wright NA, Poulsom R, Stamp G, Van Norden S, Sarraf C, Elia G, et al. Trefoil peptide gene expression in gastrointestinal epithelial cells in inflammatory bowel disease. Scand J Gastroenterol Suppl. 1992;193:76-82.
14. Alarfaj SJ, Mostafa SA, Negm WA, El-Masry TA, Kamal M, Elsaeed M, et al. Mucosal Genes Expression in Inflammatory Bowel Disease Patients: New Insights. Pharmaceuticals (Basel). 2023;16(2):324.
15. Aamann L, Vestergaard EMGronbaek H. Trefoil factors in inflammatory bowel disease. World J Gastroenterol. 2014;20(12):3223-30.
16. Philippeit C, Busch MDunker N. Epigenetic control of trefoil factor family (TFF) peptide expression in human retinoblastoma cell lines. Cell Physiol Biochem. 2014;34(3):1001-14.
17. Vestergaard EM, Nexo E, Torring N, Borre M, Orntoft TFSorensen KD. Promoter hypomethylation and upregulation of trefoil factors in prostate cancer. Int J Cancer. 2010;127(8):1857-65.
18. Lo PK, Watanabe H, Cheng PC, Teo WW, Liang X, Argani P, et al. MethySYBR, a novel quantitative PCR assay for the dual analysis of DNA methylation and CpG methylation density. J Mol Diagn. 2009;11(5):400-14.
19. Li Y, Deuring J, Peppelenbosch MP, Kuipers EJ, de Haar Cvan der Woude CJ. STAT1, STAT6 and

adenosine 3',5'-cyclic monophosphate (cAMP) signaling drive SOCS3 expression in inactive ulcerative colitis. Mol Med. 2012;18(1):1412-9.
20. Schmittgen TDLivak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. 2008;3(6):1101-8.
21. Husseiny MI, Kuroda A, Kaye AN, Nair I, Kandeel FFerreri K. Development of a quantitative methylation-specific polymerase chain reaction method for monitoring beta cell death in type 1 diabetes. PLoS One. 2012;7(10):e47942.
22. Yamasaki S, Sakata Y, Yoshida H, Shirai S, Tanaka Y, Nakano R, et al. Shorter relapse-free period after leukocyte removal therapy in younger than older patients with ulcerative colitis. Digestion. 2019;100(4):247-53.
23. Nakov R, Velikova T, Nakov V, Ianiro G, Gerova VTankova L. Serum trefoil factor 3 predicts disease activity in patients with ulcerative colitis. Eur Rev Med Pharmacol Sci. 2019;23(2):788-94.
24. Longman RJ, Poulsom R, Corfield AP, Warren BF, Wright NAThomas MG. Alterations in the composition of the supramucosal defense barrier in relation to disease severity of ulcerative colitis. J Histochem Cytochem. 2006;54(12):1335-48.
25. Low D, Mizoguchi AMizoguchi E. DNA methylation in inflammatory bowel disease and beyond. World J Gastroenterol. 2013;19(32):5238-49.
26. Koike T, Shimada T, Fujii Y, Chen G, Tabei K, Namatame T, et al. Up-regulation of TFF1 (pS2) expression by TNF-alpha in gastric epithelial cells. J Gastroenterol Hepatol. 2007;22(6):936-42.
27. Muzes G, Molnar B, Tulassay ZSipos F. Changes of the cytokine profile in inflammatory bowel diseases. World J Gastroenterol. 2012;18(41):5848-61.
28. Dossinger V, Kayademir T, Blin NGott P. Down-regulation of TFF expression in gastrointestinal cell lines by cytokines and nuclear factors. Cell Physiol Biochem. 2002;12(4):197-206.
29. Hoffmann W. Trefoil factor family (Tff) peptides and their links to inflammation: A re-evaluation and new medical perspectives. Int J Mol Sci. 2021;22(9):4909.
30. Gonzaga IM, Soares Lima SC, Nicolau MC, Nicolau-Neto P, da Costa NM, de Almeida Simao T, et al. TFF1 hypermethylation and decreased expression in esophageal squamous cell carcinoma and histologically normal tumor surrounding esophageal cells. Clin Epigenetics. 2017;9:130.
31. Rivas-Ortiz CI, Lopez-Vidal Y, Arredondo-Hernandez LJRCastillo-Rojas G. Genetic Alterations in Gastric Cancer Associated with Helicobacter pylori Infection. Front Med (Lausanne). 2017;4:47.
32. Qian Z, Jiang Y, Shou C, Yu J, Huang D, Xie H, et al. Validation of the DNA Methylation Landscape of TFF1/TFF2 in Gastric Cancer. Cancers (Basel). 2022;14(22):5474.
33. Braga Emidio N, Brierley SM, Schroeder CIMuttenthaler M. Structure, Function, and Therapeutic Potential of the Trefoil Factor Family in the Gastrointestinal Tract. ACS Pharmacol Transl Sci. 2020;3(4):583-97.