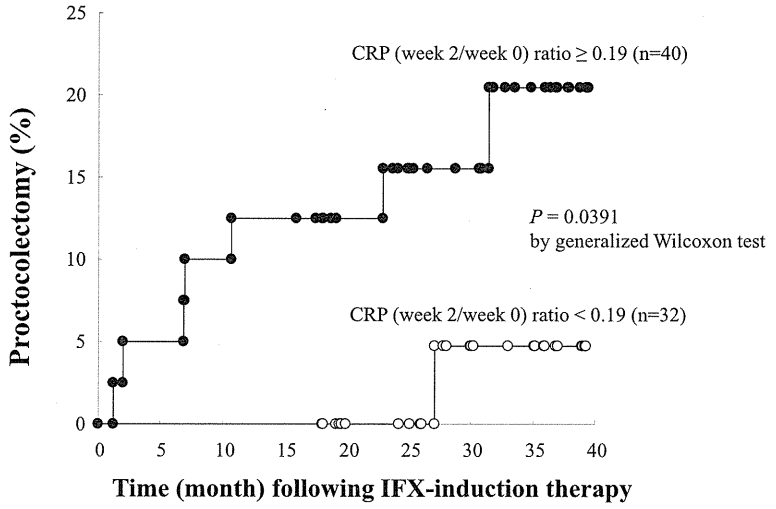

Fig. 3 The Kaplan-Meier estimator plots for the probability of proctocolectomy in subgroups of patients with cut-off value of 0.19 C-reactive protein (week 2/week 0) ratios following the initiation of infliximab-induction therapy

et al. [25] reported that blood concentration of albumin was associated with trough IFX levels and the response to IFX. We did investigate the relevance of blood albumin levels to IFX efficacy in our ROC model, but found that albumin level was not a sensitive predictor of response to IFX. However, unlike albumin, in patients with IBD, serum CRP level correlates well with disease activity and is the most widely monitored laboratory marker [9–11, 13]. Further, it has been reported that patients with UC or CD in whom CRP level decreased to normal range after completion of anti-TNF therapy had better remission maintenance time [14, 17, 18].

Unlike, TNF-α mRNA, and serum (pANCA+/ASCA-), measurement of CRP is an uncomplicated undertaking. However, the prediction of clinical response to therapy by the absolute value of CRP can be complicated due to the deviation of CRP values between different laboratories. Instead, application of the CRP ratio to judge clinical outcomes is more appropriate because CRP ratio is less affected by the differences between laboratories. The minimum detectable concentration of CRP in the method we used in this study is 0.01 mg/dL. Therefore, setting a cut-off value of 0.19 for the CRP (week 2/week 0) ratio means that patients with baseline CRP ≥ 0.05 mg/dL could be factored into the assessments. However, 5 of 72 patients had a baseline CRP value of <0.05 mg/dL. This means that the CRP (week 2/week 0) ratio will not work as the predictor for approximately 7 % of patients.

At this point, we should state specific limitations featured in this study. Firstly, the number of patients included was not large enough to allow showing stronger or otherwise weaker significance levels in our subgroup comparisons. Secondly, patients with baseline CRP value below 0.05 mg/dL could not be included in the assessment of the CRP as predictor of response to IFX. Thirdly, full endoscopy data was not included in our analyses of clinical outcomes.

Conclusion

In this study, a significant difference in CRP between IFX responders, partial-responders and non-responders during IFX induction therapy was found 2 weeks following the first IFX infusion. The differences in CRP (week 2/week0) ratios between the responders, partial-responders and non-responders were more striking than anything we had expected. This was determined by the application of an ROC model. This finding is potentially interesting in clinical setting because monitoring the CRP (week 2/week 0) ratio at an early stage like week 2 during IFX induction therapy might provide an indication to stop futile anti-TNF therapy.

Abbreviations

CRP: C-reactive protein; f-IFX: functional infliximab; IBD: Inflammatory bowel disease; IFX: Infliximab; IL: Interleukin; IQR: Interquartile range; pMayo: partial Mayo; ROC: Receiver operating characteristic; TNF-α: Tumour necrosis factor-alpha; UC: Ulcerative colitis; CD: Crohn's disease.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RI and YS: Conception, study design, and drafting of the final manuscript version; AY, KS, RF and KT: Patient management, acquisition of the data, and statistical analyses; RI, AY, KS, RF, KT and YS: Critical interpretation of the data and approval of the final manuscript version. All authors read and approved the final manuscript.

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References

1. Rutgeerts P, Sandborn WJ, Feagan BG, Reinisch W, Olson A, Johanns J, et al. Infliximab for induction and maintenance therapy for ulcerative colitis. N Engl J Med. 2005;353:2462–76.
2. Alzafiri R, Holcroft CA, Malolepszy P, Cohen A, Szilagyi A. Infliximab therapy for moderately severe Crohn's disease and ulcerative colitis: a retrospective comparison over 6 years. Clin Exp Gastroenterol. 2011;4:9–17.
3. Levin A, Shibolet O. Infliximab in ulcerative colitis. Biogeosciences. 2008;2:379–88.
4. Murch SH, Lamkin VA, Savage MO, Walker-Smith JA, MacDonald TT. Serum concentrations of tumor necrosis factor alpha in childhood chronic inflammatory bowel disease. Gut. 1991;32:913–7.
5. Braegger CP, Nicholls S, Murch SH, Stephens S, MacDonald TT. Tumor necrosis factor alpha in stool as a marker of intestinal inflammation. Lancet. 1992;339:89–91.
6. Tsukada Y, Nakamura T, Iimura M, Iizuka BE, Hayashi N. Cytokine profile in colonic mucosa of ulcerative colitis correlates with disease activity and response to granulocytapheresis. Am J Gastroenterol. 2002;97:2820–8.
7. Probert CS, Hearing SD, Schreiber S, Kühbacher T, Ghosh S, Arnott ID, et al. Infliximab in moderately severe glucocorticoid resistant ulcerative colitis: A randomised controlled trial. Gut. 2003;52:998–1002.
8. Jakobovits SL, Jewell DP, Travis SP. Infliximab for the treatment of ulcerative colitis: outcomes in Oxford from 2000 to 2006. Aliment Pharmacol Ther. 2007;25:1055–60.
9. Koelewijn CL, Schwartz MP, Samsom M, Oldenburg B. C-reactive protein levels during a relapse of Crohn's disease are associated with the clinical course of the disease. World J Gastroenterol. 2008;14:85–9.
10. Vermeire S, Van Assche G, Rutgeerts P. Laboratory markers in IBD: useful, magic, or unnecessary toys? Gut. 2006;55:426–31.
11. Haas SL, Abbatista M, Brade J, Singer MV, Böcker U. Interleukin-18 serum levels in inflammatory bowel diseases: correlation with disease activity and inflammatory markers. Swiss Med Wkly. 2009;139:140–5.
12. Feldmann M, Brennan FM, Maini RN. Role of cytokines in rheumatoid arthritis. Annu Rev Immunol. 1996;14:397–440.
13. Vermeire S, Van Assche G, Rutgeerts P. C-reactive protein as a marker for inflammatory bowel disease. Inflamm Bowel Dis. 2004;10:661–5.
14. Reinisch W, Wang Y, Oddens BJ, Link R. C-reactive protein, an indicator for maintained response or remission to infliximab in patients with Crohn's disease: a post-hoc analysis from ACCENT I. Aliment Pharmacol Ther. 2012;35:568–76.
15. Magro F, Rodrigues-Pinto E, Santos-Antunes J, Vilas-Boas F, Lopes S, Nunes A, et al. High C-reactive protein in Crohn's disease patients predicts nonresponse to infliximab treatment. J Crohns Colitis. 2014;8:129–36.
16. Sandborn WJ, Colombel JF, Enns R, Feagan BG, Hanauer SB, Lawrance IC, et al. Natalizumab induction and maintenance therapy for Crohn's disease. N Engl J Med. 2005;353:1912–25.
17. Kiss LS, Szamosi T, Molnar T, Miheller P, Lakatos L, Vincze A, et al. Early clinical remission and normalisation of CRP are the strongest predictors of efficacy, mucosal healing and dose escalation during the first year of adalimumab therapy in Crohn's disease. Aliment Pharmacol Ther. 2011;34:911–22.
18. Armuzzi A, Pugliese D, Danese S, Rizzo G, Felice C, Marzo M, et al. Infliximab in steroid-dependent ulcerative colitis: effectiveness and predictors of clinical and endoscopic remission. Inflamm Bowel Dis. 2013;19:1065–72.
19. Lewis JD, Chuai S, Nessel L, Lichtenstein GR, Aberra FN, Ellenberg JH. Use of the noninvasive components of the Mayo score to assess clinical response in ulcerative colitis. Inflamm Bowel Dis. 2008;14:1660–6.
20. Sandborn WJ, van Assche G, Reinisch W, Colombel JF, D'Haens G, Wolf DC, et al. Adalimumab induces and maintains clinical remission in patients with moderate-to-severe ulcerative colitis. Gastroenterology. 2012;142:257–65.
21. Yamada A, Sono K, Hosoe N, Takada N, Suzuki Y. Monitoring functional serum antitumor necrosis factor antibody level in Crohn's disease patients who maintained and those who lost response to anti-TNF. Inflamm Bowel Dis. 2010;16:1898–904.
22. Cornillie F, Shealy D, D'Haens G, Geboes K, Van Assche G, Ceuppens J, et al. Infliximab induces potent anti-inflammatory and local immunomodulatory activity but no systemic immune suppression in patients with Crohn's disease. Aliment Pharmacol Ther. 2001;15:463–73.
23. Olsen T, Goll R, Cui G, Christiansen I, Florholmen J. TNF-alpha gene expression in colorectal mucosa as a predictor of remission after induction therapy with infliximab in ulcerative colitis. Cytokine. 2009;46:222–7.
24. Ferrante M, Vermeire S, Katsanos KH, Noman M, Van Assche G, Schnitzler F, et al. Predictors of early response to infliximab in patients with ulcerative colitis. Inflamm Bowel Dis. 2007;13:123–8.
25. Fasanmade AA, Adedokun OJ, Olson A. Serum albumin concentration: a predictive factor of infliximab pharmacokinetics and clinical response in patients with ulcerative colitis. Int J Clin Pharmacol Ther. 2010;48:297–308.

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ORIGINAL ARTICLE

Randomized Clinical Trial

Effectiveness of probiotic therapy for the prevention of relapse in patients with inactive ulcerative colitis

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Author contributions: Yasushi Y and Yasuo S contributed equally to this work; Yasushi Y and Yasuo S designed research; Yasushi Y, Akihiro Y, Ryuichi F, Koji S, Aisaku O, Kentaro N, Hiroshi A, Yukiko T, Nobuo H and Nobuo T performed research; Yasushi Y and Yasuo S contributed new reagents/analytic tools; Yasushi Y and Yasuo S analyzed data; and Yasushi Y and Yasuo S wrote the paper.

Ethics approval: The study was reviewed and approved by the ethical committee of Toho University Sakura Medical Center.

Clinical trial registration: This study is not registered at any URL.

Informed consent: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest: Yasushi Yoshimatsu has no conflict of interest.

Data sharing: No additional data are available.

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Abstract

AIM: To evaluate the effectiveness of probiotic therapy for suppressing relapse in patients with inactive ulcerative colitis (UC).

METHODS: Bio-Three tablets, each containing 2 mg of lactomin (*Streptococcus faecalis* T-110), 10 mg of *Clostridium butyricum* TO-A, and 10 mg of *Bacillus mesentericus* TO-A, were used as probiotic therapy. Sixty outpatients with UC in remission were randomly assigned to receive 9 Bio-Three tablets/day (Bio-Three group) or 9 placebo tablets/day (placebo group) for 12 mo in addition to their ongoing medications. Clinical symptoms were evaluated monthly or on the exacerbation of symptoms or need for additional medication. Fecal samples were collected to analyze bacterial DNA at baseline and 3-mo intervals. Terminal restriction fragment length polymorphism and cluster analyses were done to examine bacterial components of the fecal microflora.

RESULTS: Forty-six patients, 23 in each group, completed the study, and 14 were excluded. The relapse rates in the Bio-Three and placebo groups were respectively 0.0% *vs* 17.4% at 3 mo (P = 0.036), 8.7% *vs* 26.1% at 6 mo (P = 0.119), and 21.7% *vs* 34.8% (P = 0.326) at 9 mo. At 12 mo, the remission rate was 69.5% in the Bio-Three group and 56.6% in the placebo group (P = 0.248). On cluster analysis of fecal flora, 7 patients belonged to cluster Ⅰ, 32 to cluster Ⅱ, and 7 to cluster Ⅲ.

CONCLUSION: Probiotics may be effective for

maintaining clinical remission in patients with quiescent UC, especially those who belong to cluster I on fecal bacterial analysis.

Key words: Ulcerative colitis; Probiotics; Inflammatory bowel disease; Cluster analysis

Core tip: We conducted a single-center, randomized, double-blind, placebo-controlled study to examine whether 12 mo of probiotic therapy was useful for preventing relapse of ulcerative colitis (UC) in patients who were already in remission. The relapse rates in the probiotic therapy group and placebo group were respectively 0.0% *vs* 17.4% at 3 mo (P = 0.036), 8.7% *vs* 26.1% at 6 mo (P = 0.119), and 21.7% *vs* 34.8% (P = 0.326) at 9 mo. At 12 mo, the remission rate was 69.5% in the probiotic therapy group and 56.6% in the placebo group (P = 0.248). Therefor probiotics may be effective for maintaining clinical remission in patients with quiescent UC.

Yoshimatsu Y, Yamada A, Furukawa R, Sono K, Osamura A, Nakamura K, Aoki H, Tsuda Y, Hosoe N, Takada N, Yasuo Suzuki Y. Effectiveness of probiotic therapy for the prevention of relapse in patients with inactive ulcerative colitis. *World J Gastroenterol* 2015; 21(19): 0000-0000 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i19/0000.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i19.0000

INTRODUCTION

Ulcerative colitis (UC) is a chronic, idiopathic, refractory, inflammatory bowel disease (IBD) characterized by inflammatory mucosal injury of the colon, with repeated periods of remission and relapse. The cause and etiology of UC remain unclear. The mainstay of treatment for UC is sulfasalazine- or mesalazine-based therapy. In patients with moderate to severe UC, steroids are used concurrently to attempt to induce remission. However, a considerable number of cases are resistant to steroids. Patients with steroid-resistant disease are given immunosuppressants and newly developed biological preparations to promote remission induction. Although these new treatments have enhanced the remission induction rate as compared with conventional therapy, achievement of a high long-term rate of remission maintenance remains a largely unattained goal. Steroids are very effective for the induction of remission, but do not contribute to remission maintenance. In addition, long-term treatment with high doses of steroids is associated with high rates of various adverse effects, seriously impairing the quality of life of patients. Sulfasalazine, mesalazine, and immunomodulators promote remission maintenance, but are not adequately effective. Moreover, an appreciable number of patients cannot tolerate these drugs, and immunomodulators can cause serious adverse events, necessitating close follow-up. Therefore, the development of new remission maintenance treatments that are very effective and safe with good compliance when used on a long-term basis has been eagerly awaited.

Recently, probiotic therapy has been acknowledged to be potentially effective and safe in patients with UC. Probiotics are defined as a live microbial feed nutritional supplement that beneficially affects the host by improving the balance of the intestinal flora. Studies of animal models of colitis have suggested that the intestinal flora has an important role in the pathogenesis of colitis. In IBD-sensitive knockout or transgenic mice, colitis develops in the presence of a normal intestinal flora, but not in mice raised in a germ-free environment, strongly suggesting that the intestinal flora participates in the development of colitis[1,2]. Therefore, probiotic therapy designed to correct the intestinal flora is expected to be useful for preventing colitis.

Many studies have examined the effects of specific bacterial strains or species in active UC. However, very few studies have reported on the relation between the intestinal flora as a whole (including microorganisms that are difficult or impossible to culture) and the pathological characteristics of UC[3-7]. In a previous study, we therefore gave probiotic or synbiotic therapy for 4 wk to 20 patients with mild to moderate UC who did not respond to, or could not tolerate, standard therapy [oral mesalamine preparations, sulfasalazine, azathiopurine (AZA)/6-mercaptopurine (6-MP), and mesalamine enemas]. Our results confirmed that such therapy can improve clinical symptoms and endoscopic findings and provided evidence that remission induction is promoted by a certain improvement in the intestinal flora. We also reported that probiotic therapy might be effective for maintenance of remission[8]. On the basis of the results of our previous study, we conducted a single-center, randomized, double-blind, placebo-controlled study to examine whether 12 mo of probiotic therapy is useful for preventing relapse of UC in patients who were already in remission.

MATERIALS AND METHODS

Patients

The study group comprised patients with UC in remission who were receiving treatment on an outpatient basis at Sakura Medical Center, Toho University. UC was diagnosed in accordance with the diagnostic criteria proposed by the Survey Research Group of Intractable Inflammatory Intestinal Disorders/ Specified Diseases, Japanese Ministry of Health, Labour and Welfare. Patients 13 years or older in whom the CAI was maintained at 5 or less while receiving drugs

such as mesalazine, salazosulfapyridine, or steroids, with no change in treatment regimens within 4 wk before study entry, were enrolled in this randomized, double-blind, placebo-controlled study.

Patients were excluded if they had serious cardiac disease, serious renal disease, hypotension (systolic blood pressure, ≤ 80 mmHg), a history of shock during extracorporeal circulation, serious infections such as sepsis or pneumonia, or a serum hemoglobin concentration of less than 10 g/dL. We also excluded patients who newly began treatments such as leukocytapheresis, granulocyte adsorptive apheresis, or immunosuppressant therapy with drugs such as 6-mercaptopurine, azathioprine, and cyclosporine to improve symptoms, as well as patients who had milk allergy or a CAI of 6 or higher. Pregnant women were also excluded. All procedures were in accordance with the Helsinki Declaration of 1975, as revised in 1983.

Study probiotic

Bio-Three tablets (Toa Pharmaceutical Co., Ltd., Toyama, Japan), a live microbial preparation, and matching placebo tablets (Toa Pharmaceutical Co., Ltd.) were used as the study preparations. Bio-Three tablets were granted manufacturing approval in 1963. Each tablet contains 2 mg of lactomin (*Streptococcus faecalis* T-110), 10 mg of *Clostridium*, (*Clostridium butyricum* TO-A), and 10 mg of *Bacillus* (*Bacillus mesentericus* TO-A), combined with potato starch and lactose. This preparation is effective for resolving various symptoms caused by abnormal intestinal flora and mainly improves bowel movement disorders. Placebo tablets were prepared by substituting equivalent amounts of starch for the probiotic powder. Placebo tablets were identical to Bio-Three tablets and could not be distinguished from the active preparation on the basis of appearance.

Study design and treatment

At the start of the study, 30 outpatients were randomly assigned to the Bio-Three group and 30 to the placebo group by means of a computer-generated scheme. The study protocol was reviewed and approved by the Ethics Committee of Sakura Medical Center, Toho University.

In both the Bio-Three group and the placebo group, patients orally received 3 tablets 3 times daily. In principle, the duration of treatment was 12 mo. Fecal samples were collected immediately before and 3, 6, 9, and 12 mo after the start of treatment. Fecal samples for measurement of organic acids were preserved by freezing, without modification. Fecal samples used for DNA extraction were suspended in GTC solution (100 mmol/L Tris-HCl, pH 9.0; 40 mmol/L Tris-EDTA, pH 8.0; and 4 mol/L guanidine thiocyanate) and were preserved at 4 ℃. As for concomitant medication (therapy), the use of mesalazine and salazosulfapyridine was unrestricted, but steroids could not be used as remission maintenance therapy. The use of drugs with similar effects as the study drug, potentially affecting the evaluation of effectiveness (*i.e.*, other active live microbial preparations, laxatives, *etc.*) was prohibited from 1 wk before study entry to the completion of the study. In principle, the use of oral antibiotics was also prohibited, but the use of topical antibiotics other than oral preparations was not particularly restricted. If a patient received a new treatment in addition to their basic therapy with drugs such as mesalazine or salazosulfapyridine, relapse was diagnosed, and the study treatment and fecal sample collection were discontinued.

Analysis of intestinal microflora

DNA extraction: About 800 μL of the fecal sample suspension preserved at 4 ℃ was transferred to a tube containing zirconia beads (Nippon Gene Co., Ltd., Tokyo, Japan), and the cells were processed with the use of FastPrep FP120A cell disruptor (MP Biomedicals, Irvine, CA). After cooling on ice, the specimen was centrifuged at 5000 rpm for 1 min. DNA was automatically extracted from the processed supernatant with the use of a 12GC and GC series Magtration-MagaZorb DNA Common Kit 200N (Precision System Science, Chiba, Japan). The final concentration of the extracted DNA was adjusted to 10 ng/μL.

Terminal restriction fragment length polymorphism analysis: Terminal restriction fragment length polymorphism (T-RFLP) analysis was performed as described by Nagashima *et al*[9]. The 16S rRNA gene was amplified with the use of primer sets516F (5′-TGCCAGCAGCCGCGGTA-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The 5′-end of the forward primer 516F was labeled with 6′-carboxyfluorescein. Amplified polymerase chain reaction (PCR) products were refined with the use of MultiScreen® PCRμ96 filter plates (Millipore, Tokyo, Japan).

The refined PCR products (about 3 μL) were digested for 3 h at 55 ℃ with 10 U of *Bsl* I restriction enzyme (New England Biolabs, Inc., Ipswich, MA, United States). The length of the separated fluorescent PCR fragment was determined with an ABI PRISM 3130xl genetic analyzer (Applied Biosystems, Tokyo, Japan), and the data were analyzed with GeneMapper® software. MapMarker® X-Rhodamine Labeled 50-1000bp (BioVentures, Inc., Murfreesboro, TN, United States) was used as a size standard marker.

Cluster analysis: To objectively interpret differences in T-RFLP patterns, NTSYSpc software (Exeter Software, Setauket, NY, United States) was used to perform cluster analysis. Each terminal restriction fragment (T-RF) was expressed as a percentage of the peak area of all T-RFs. Disparity in similarity

Table 1 Baseline characteristics of the study group

	Bio-three (n = 23)	Placebo (n = 23)
Male/female	16/7	12/11
Age (yr, mean ± SD)	44.8 ± 13.8	42.9 ± 15.9
Age of onset (yr)	37.1 ± 14.4	36.0 ± 14.2
Disease duration (yr, mean ± SD)	8.0 ± 6.3	6.7 ± 5.9
Left colon	6	9
Proctosigmidis	6	5
Total/subtotal	11	9
Concomitant drug		
Pentasa	11	13
Salazopirin	10	9
Pentasa + salazopirin	1	0
Nothing	1	1

among fecal samples in individual patients was calculated using a correlation matrix and was presented graphically on tree diagrams with the use of a weighted pair-group method with arithmetic mean (WPGMA) clustering[10].

High-performance liquid chromatography analysis of fecal organic acids: Organic acid concentrations in fecal samples were measured as follows. About 0.1 g of fecal sample was measured and combined with trans-crotonic acid as an internal standard substance, and extraction was performed twice with 0.6 mL of 0.25% ammonia solution. After adding a 0.3-fold dilution of 10% (w/v) perchloric acid, deproteinization was performed by centrifugation. The solution was filtered and analyzed with a post-column high-performance liquid chromatography (HPLC) system (Waters, Milford MA, United States). Organic acids in the sample were separated with an ion exchange column (organic acid column, 7.8 mm i.d. x 300 mm long; Waters). The reaction temperature in the column and post-column was 60 ℃. The mobile phase was 0.08% perchloric acid delivered at a flow rate of 0.8 mL/min. The solution eluted from the column was allowed to react with BTB solution (0.2 mmol/L bromothymol blue, 5.2 mmol/L sodium hydroxide, and 15 mmol/L disodium hydrogen phosphate), delivered at a flow rate of 0.8 mL/min. The absorbance was quantitatively measured at 445 nm using an ultraviolet visible spectrophotometer (2487 Dual λ UV/Vis Detector; Waters).

Statistical analysis

Clinical data were statistically analyzed with SAS software (version 8.2, SAS Institute Inc., Cary, NC, United States). For patients with UC in remission, the Kaplan-Meier method was used to compare the cumulative non-relapse rate over the course of 12 mo between the Bio-Three group and placebo group. The statistical significance of differences between groups was evaluated with the log-rank test and the generalized Wilcoxon test, and 95% confidence intervals were calculated.

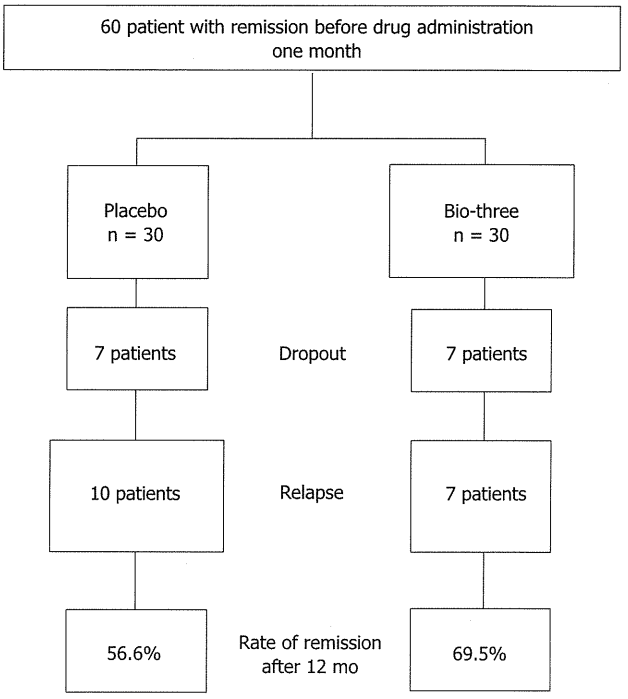


Figure 1 Clinical outcomes of patients according to treatment received.

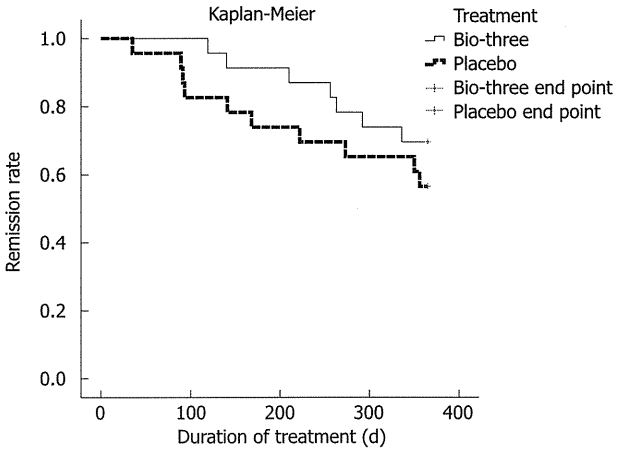
RESULTS

Patient characteristics

At the start of the study, 30 patients each were randomly assigned to the Bio-Three group and the placebo group. After randomization, the baseline characteristics of sex, age, age at disease onset, disease duration, disease extent, and concomitant treatment did not differ between the groups (Table 1). Among the enrolled subjects, 7 patients in each group were excluded because they met the exclusion criteria specified in the protocol, such as the use of prohibited drugs or refusal to participate in the study. Treatment was actually begun in 23 patients in the Bio-Three group and 23 in the placebo group (Figure 1).

Clinical results

After the study began, the number of patients who had relapse was 2 at 6 mo, 5 at 9 mo, and 7 at 12 mo among the 23 patients in the Bio-Three group and 4 at 3 mo, 6 at 6 mo, 8 at 9 mo, and 10 at 12 mo among the 23 patients in the placebo group. Kaplan-Meier curves were plotted, and relapse rates at each time point were compared between the groups with the use of the χ^2 test (two-tailed, α = 0.05). The P value was 0.0363 at 3 mo, 0.1197 at 6 mo, and 0.3259 at 9 mo. The cumulative remission maintenance rate at 12 mo was 56.6% (n = 12) in the placebo group and 69.5% (n = 16) in the Bio-Three group (Figure 2). When remission maintenance rates were compared between the treatment groups with the use of the log-rank test, the P value was 0.302, with a hazard ratio of 0.607 (95%CI: 0.23-1.59). On the generalized Wilcoxon test, the P value was 0.248. During our study, no adverse



	Chi-square test	Degree of freedum	Significance probability
Log rank (Mantel-Cox)	1.007	1	0.316

Figure 2 Cumulative remission maintenance rate in the Bio-Three group and placebo group.

changes were observed in the Bio-Three group, as compared with the placebo group, indicating that there is no problem with the safety of Bio-Three.

Bacteriological analysis

In our previous study evaluating the effectiveness of Bio-Three for inducing remission in patients with active UC[8], a cluster analysis was performed to evaluate fluctuations in fecal flora, using the T-RF data derived by digestion of the PCR products with *BsI* I restriction enzyme, and several findings were obtained. In the present study of the effectiveness of Bio-Three for remission maintenance, the PCR products were similarly digested with *BsI* I restriction enzyme, and cluster analysis of the T-RF data showed that the fecal flora can be divided into 3 clusters, consistent with the results of our previous study. To confirm whether the 3 clusters in the present study correspond to the 3 clusters in our previous study, the fecal sample data used in our previous study of remission induction were linked to the fecal sample data in the present study, and another cluster analysis was performed. The results showed that each of the fecal samples from our previous study belonged to the same clusters as those in our previous analysis. Therefore, for convenience the same names of the clusters in our previous study were used in the present study, *i.e.*, the cluster in the upper part of the figure was named cluster Ⅱ, the cluster in the middle part was named cluster Ⅲ, and the other cluster was named cluster Ⅰ (Figure 3, Table 2).

A total of 138 fecal samples belonged to cluster Ⅱ. The clinical outcomes of the 32 subjects who belonged to cluster Ⅱ at the start of treatment were remission maintained in 11 of the 16 patients in the Bio-Three group and 10 of the 16 patients in the placebo group. This difference was not significant. As a characteristic of T-RF in cluster Ⅱ, OTU124 accounted for a significantly higher proportion of total T-RF peak area in cluster Ⅱ than in the other clusters.

A total of 28 fecal samples belonged to cluster Ⅲ. The clinical outcomes of the 7 subjects who belonged to cluster Ⅲ at the start of treatment were remission maintained in 2 of the 4 patients in the Bio-Three group and 2 of the 3 patients in the placebo group. The difference between the groups was not significant.

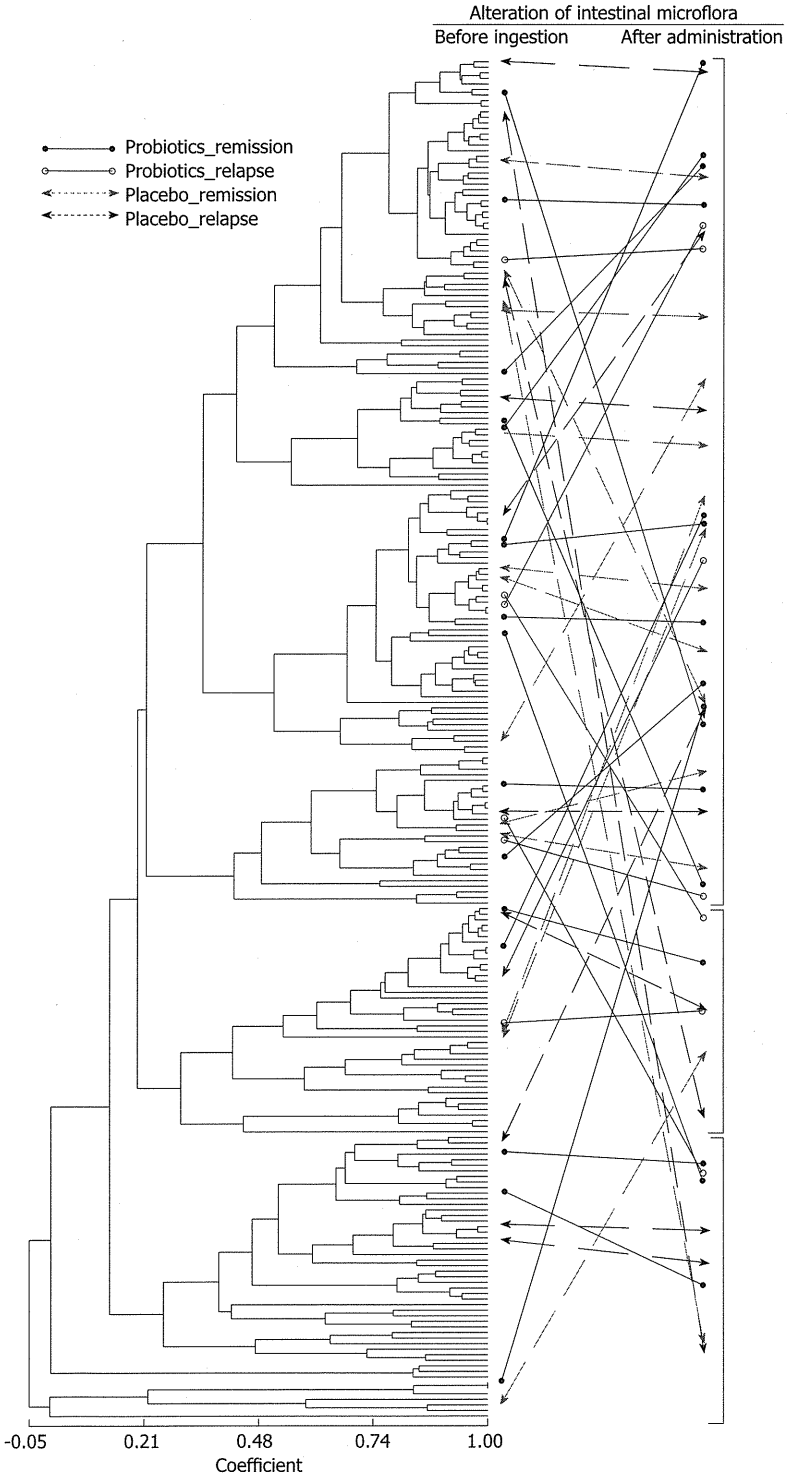
A total of 39 fecal samples belonged to cluster Ⅰ. The clinical outcomes in the 7 subjects who belonged to cluster Ⅰ at the start of treatment were remission maintained in all 3 patients in the Bio-Three group, as compared with only 1 of the 4 patients in the placebo group. Among the 39 fecal samples that belonged to cluster Ⅰ, 19 fecal samples were derived from patients in the Bio-Three group, and 17 of these samples were from patients who had a final evaluation of remission maintained. The other 20 fecal samples were from patients in the placebo group, and 8 of these samples were from patients who had a final evaluation of remission maintained. When these data were compared with the use of Pearson's χ^2 test, the *P* value was 0.0013, indicating that the rate of remission maintenance was significantly higher in the Bio-Three group than in the placebo group, and the relapse rate was significantly lower in the Bio-Three group than in the placebo group.

HPLC analysis of fecal organic acids

The fecal concentrations of short-chain fatty acids did not differ significantly between the Bio-Three group and the placebo group at any time during treatment. On comparison of the clusters derived by T-RFLP analysis, the ratio of the concentration (mmol/L) of butyrate to that of acetate (Bu/Ac ratio) was significantly higher in cluster Ⅰ than in clusters Ⅱ and Ⅲ. When fecal organic acids were compared according to clinical outcomes (*i.e.*, between fecal samples obtained at each of the specified times from patients in whom remission was maintained for 1 year and fecal samples from patients who had relapse within 6 mo after fecal collection), the concentrations of butyric acid and other short-chain fatty acids did not differ significantly between the groups. However, the Bu/Ac ratio at each of the sampling times was significantly higher in fecal samples obtained from patients who had relapse within 6 mo after fecal collection than in those obtained from patients who remained in remission (Table 3).

DISCUSSION

T-RFLP is a molecular technique that allows the diversity and colony structure of microbial complexes to be promptly compared and the diversity of ecosystems to be evaluated[11,12]. Recently, several studies have performed T-RFLP in patients with UC.



Cluster Ⅱ	No. of patients (at the start)	Clinical effectiveness (after treatment)
Probiotics	16	Remission: 11 Relapse: 5
Placebo	16	Remission: 10 Relapse: 6

Cluster Ⅲ	No. of patients (at the start)	Clinical effectiveness (after treatment)
Probiotics	4	Remission: 2 Relapse: 2
Placebo	3	Remission: 2 Relapse: 1

Cluster Ⅰ	No. of patients (at the start)	Clinical effectiveness (after treatment)
Probiotics	3	Remission: 3 Relapse: 0
Placebo	4	Remission: 1 Relapse: 3

Figure 3 Alteration of intestinal microflora after treatment in the Bio-Three group and placebo group. The dendrogram indicates the similarity of individual intestinal microflora T-RFLP patterns of fecal samples obtained after 0, 3, 6, 9, and 12 mo of treatment. Solid line and circles, Bio-Three group and remission; double line and open circles, Bio-Three group and relapse; solid dotted line and arrows, placebo group and remission; double dotted line and arrows, placebo group and relapse. The first and last samples from the same individuals are connected with lines. The tables indicate clinical effectiveness according to the cluster that the subjects belonged to at the start of this study.

However, few studies have examined time-series samples obtained from the same subjects. Most previous investigations have evaluated fecal samples obtained at a specific time[13-17].

We previously studied the effectiveness of probiotic therapy for inducing remission in patients with flare-ups of UC. On T-RFLP cluster analysis, fecal flora could be divided into 3 clusters, designated as clusters Ⅰ, Ⅱ, and Ⅲ. The flora of patients whose UC disease activity index (UCDAI) was improved by probiotic therapy was centered around cluster Ⅱ. Cluster Ⅱ flora was characterized by a high ratio of OTU124[8].

Table 2 Comparison of characteristics of fecal samples (according to cluster)

Group (sample number)	OTU124/All T-RFs (%)	Butyrate/acetate ratio (mo)
Cluster I	2.8 ± 3.6	0.254 ± 0.172
Cluster II (n = 138)	27.6 ± 12.0	0.184 ± 0.147
Cluster III (n = 28)	18.0 ± 10.8	0.075 ± 0.126

Data are expressed as means ± SD. Because equal variances were rejected for each variable, multiple comparisons were performed with Dunnett's T3 test. Different letters indicate the presence of a significant difference (P < 0.05). T-RF: Terminal restriction fragment.

Table 3 Comparison of characteristics of fecal samples (according to clinical outcomes)

Group (sample number)	OTU124/All T-RFs (%)	Butyrate/Acetate ratio (mol)
Remission (before treatment) (n = 29)	22.7 ± 13.8	0.165 ± 0.143
Remission (months 3 and 6) (n = 58)	22.0 ± 14.6	0.145 ± 0.136
Remission (months 9 and 12) (n = 56)	21.7 ± 14.1	0.160 ± 0.146
Relapse (within 6 mo) (n = 28)	20.7 ± 14.7	0.260 ± 0.185

Data are expressed as means ± SD. Because each variable had equal variances, multiple comparisons were performed with Tukey's honestly significant difference test. Different letters indicate the presence of a significant difference (P < 0.05). "Relapse (within 6 mo)" means that fecal samples were obtained within 6 mo before relapse (*i.e.*, relapse occurred within 6 mo after collection of fecal samples).

Nagashima *et al*[9], who proposed the T-RFLP technique used in our study, reported that OTU124 is derived from *Bifidobacteria*. Many studies have examined the relation between *Bifidobacteria* and UC, and most have reported that *Bifidobacteria* mitigates inflammation associated with UC[18-21]. On the basis of these findings, we previously concluded that cluster II flora is "healthy intestinal flora (appropriate intestinal flora)" in patients with UC. Probiotic therapy may therefore be less effective for improving flora in patients who initially have cluster II flora, leading only to marginal improvement clinically[8].

The results of the present study similarly suggest that cluster II represents "healthy intestinal flora." Consequently, probiotic therapy is expected to be of the greatest potential benefit for intestinal flora that is most dissimilar to cluster II. This speculation is supported by the following two points. First, in our previous study of patients with "flare-ups" of UC, only 6 (30%) of the 20 subjects had intestinal flora belonging to cluster II before treatment[8]. In the present study of patients with UC "after induction of remission," 32 (70%) of the 46 subjects had intestinal flora belonging to cluster II. Given that the proportion of patients with "appropriate intestinal flora" is expected to be higher among patients with remission induction and remission maintenance than among those with relapse, the fact that the majority of patients in the present study, who were already in remission, belonged to cluster II supports our speculation that probiotic therapy is potentially most beneficial for patients with intestinal flora most dissimilar to cluster II. Second, among the 32 subjects who belonged to cluster II at the start of treatment in the present study, remission was maintained in 11 of the 16 patients in the Bio-Three group and 10 of the 16 patients in the placebo group. There was no difference in the remission maintenance rate between these groups. In contrast, among the 7 subjects who belonged to cluster I (*i.e.*, the cluster farthest from cluster II) before treatment, remission was maintained in all 3 patients in the Bio-Three group, whereas relapse occurred in 3 of the 4 patients in the placebo group. These findings indicate that probiotic therapy was less effective in patients who initially belonged to cluster II ("appropriate intestinal flora"), with no difference from placebo. In contrast, probiotic therapy was potentially most beneficial for patients with intestinal flora belonging to cluster I flora before treatment, the cluster that is farthest from cluster II. Consequently, probiotic therapy was more effective than placebo for maintaining remission in this subgroup of patients. These results were very interesting and are consistent with the findings of our previous study[8].

The fecal Bu/Ac ratio differed between patients with relapse and those in whom remission was maintained for 12 mo and was significantly higher within 6 mo before relapse than at other times (Table 3). Interestingly, the Bu/Ac ratio tended to be higher in feces belonging to cluster I than in the other clusters (Table 2). These findings may be attributed to the following mechanism. Butyrate serves as an energy source for intestinal epithelial cells and is known to induce apoptosis of colorectal cancer cells and the differentiation of intestinal epithelial cells. In addition, butyrate has been shown to inhibit the activation of nuclear factor kappa B (NF-κB) and to have anti-inflammatory properties[11,22,23]. On the other hand, the utilization efficiency of butyrate has been reported to be low in the colonic mucosa of patients with refractory UC. The anti-inflammatory activity of butyrate has prompted several studies of its effectiveness and mechanism of action in patients with UC[24-28].

In our previous study evaluating the effectiveness of Bio-Three for inducing remission in patients with UC, the decrease in the UCDAI after treatment (*i.e.*, the improvement in symptoms of UC) correlated with the decrease in the fecal butyrate concentration[8]. On the basis of this finding, we performed breath tests after administration of [1-13C]-butyrate enemas in 10 patients with active UC and 12 with quiescent UC and confirmed that the utilization efficiency of butyrate was decreased in patients with high inflammatory activity[29-33]. These findings suggest that an increased Bu/Ac ratio resulting from decreased absorption of butyrate, an indicator of anti-inflammatory activity,

and an increase in fecal butyrate concentrations is associated with a higher risk of relapse in patients with UC. The mean rate of remission maintenance at 12 mo in patients who receive mesalazine preparations alone is estimated to be about 61% (range: 45%-71%) on the basis of the results of previous randomized controlled trials[34-41]. This rate is similar to the relapse rate among patients who received mesalazine preparations alone (56.6%) in the placebo group of our study.

We used the Kaplan-Meier method to compare relapse rates between the treatment groups. The relapse rate after 12 mo did not differ significantly between the Bio-Three group and the placebo group on either the log-rank test or generalized Wilcoxon test. However, detailed analysis showed that clinical effectiveness differed between the Bio-Three group and placebo group among patients who belonged to cluster Ⅰ. As mentioned above, probiotic therapy is most likely to be effective in patients with intestinal flora belonging to cluster Ⅰ, which is characterized by both a low ratio of OTU124 (which tended to be high in patients belonging to cluster Ⅱ, classified as "healthy intestinal flora") and a high fecal Bu/AC ratio (which was significantly higher in patients within 6 mo before relapse than in patients without relapse).

Combining probiotics or synbiotics with conventional drugs has been recommended as a safe and effective treatment for patients with active UC. For more than 10 years considerable attention has focused on the effectiveness of probiotic therapy for UC[42]. Nearly all studies have reported that probiotics such as VSL#3[43-45], BIFICO[46], and *E. coli* Nissle 1917[47] and prebiotics such as GBF[48-50] and BGS[51] are useful for maintaining remission maintenance and preventing relapse in patients with UC. In comprehensive Cochrane reviews of clinical studies evaluating the effectiveness of probiotics for UC, Mallon *et al*[52] and Naidoo *et al*[53] concluded that although probiotics are ineffective for the induction of remission, probiotics combined with conventional therapy are expected to be effective for maintenance of remission. A meta-analysis conducted by Sang *et al*[54]. reported that probiotics are slightly but not significantly effective for remission induction, but significantly contribute to remission maintenance.

The results of our study suggest that cluster analysis of patients' intestinal flora before treatment can contribute to the effective use of probiotic therapy. To our knowledge, our study represents an unprecedented attempt to define factors related to the effectiveness of Bio-Three for the prevention of relapse in patients with inactive UC. Not only the fecal flora, but also the fecal concentration of short-chain fatty acids differed between patients who had relapse within 1 year and those in whom remission was maintained for 1 year. This finding suggests that patient profiling on the basis of factors such as the results of cluster analysis of fecal flora and the fecal short-chain fatty acid concentration might facilitate prediction of the response to treatment and future clinical status in patients with UC.

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COMMENTS

Background

Ulcerative colitis (UC) is a chronic, idiopathic, refractory, inflammatory bowel disease (IBD) characterized by inflammatory mucosal injury of the colon, with repeated periods of remission and relapse. Recently, probiotic therapy has been acknowledged to be potentially effective and safe in patients with UC. Probiotics are defined as a live microbial feed nutritional supplement that beneficially affects the host by improving the balance of the intestinal flora.

Research frontiers

Studies of animal models of colitis have suggested that the intestinal flora has an important role in the pathogenesis of colitis. In IBD-sensitive knockout or transgenic mice, colitis develops in the presence of a normal intestinal flora, but not in mice raised in a germ-free environment, strongly suggesting that the intestinal flora participates in the development of colitis. Therefore, probiotic therapy designed to correct the intestinal flora is expected to be useful for preventing colitis.

Innovations and breakthroughs

The results confirmed that probiotic therapy can improve clinical symptoms and endoscopic findings and provided evidence that remission induction is promoted by a certain improvement in the intestinal flora. The authors also reported that probiotic therapy might be effective for maintenance of remission.

Applications

The authors conducted a single-center, randomized, double-blind, placebo-controlled study to examine whether 12 mo of probiotic therapy is useful for preventing relapse of UC in patients who were already in remission.

Peer-review

In this study, the authors designed the randomized double-blind controlled study to evaluate the effectiveness of probiotic therapy for suppressing relapse in patients with UC. They concluded that probiotics may be effective for maintaining clinical remission in patients with quiescent UC, especially those who belong to cluster Ⅰ on fecal bacterial analysis. This paper is well written and the results of the study are clinically interesting.

REFERENCES

1 **Furrie E**, Macfarlane S, Kennedy A, Cummings JH, Walsh SV, O'neil DA, Macfarlane GT. Synbiotic therapy (Bifidobacterium longum/Synergy 1) initiates resolution of inflammation in patients with active ulcerative colitis: a randomised controlled pilot trial. *Gut* 2005; **54**: 242-249 [PMID: 15647189 DOI: 10.1136/gut.2004.044834]

2 **Campieri M**, Gionchetti P. Bacteria as the cause of ulcerative colitis. *Gut* 2001; **48**: 132-135 [PMID: 11115835 DOI: 10.1136/gut.48.1.132]

3 **Danese S**, Sans M, Fiocchi C. Inflammatory bowel disease: the role of environmental factors. *Autoimmun Rev* 2004; **3**: 394-400 [PMID: 15288007 DOI: 10.1016/j.autrev.2004.03.002]

4 **Macfarlane GT**, Furrie E, Macfarlane S. Bacterial milieu and mucosal bacteria in ulcerative colitis. *Novartis Found Symp* 2004; **263**: 57-64; discussion 64-70, 211-8 [PMID: 15669634]

5 **Cummings JH**, Kong SC. Probiotics, prebiotics and antibiotics in inflammatory bowel disease. *Novartis Found Symp* 2004; **263**: 99-111; discussion 111-4, 211-8 [PMID: 15669637]

6 **Ohkusa T**, Okayasu I, Ogihara T, Morita K, Ogawa M, Sato N. Induction of experimental ulcerative colitis by Fusobacterium varium isolated from colonic mucosa of patients with ulcerative colitis. *Gut* 2003; **52**: 79-83 [PMID: 12477765 DOI: 10.1136/gut.52.1.79]

7 **Kanauchi O**, Matsumoto Y, Matsumura M, Fukuoka M, Bamba T. The beneficial effects of microflora, especially obligate anaerobes, and their products on the colonic environment in inflammatory bowel disease. *Curr Pharm Des* 2005; **11**: 1047-1053 [PMID: 15777254 DOI: 10.2174/1381612053381675]

8 **Tsuda Y**, Yoshimatsu Y, Aoki H, Nakamura K, Irie M, Fukuda K, Hosoe N, Takada N, Shirai K, Suzuki Y. Clinical effectiveness of probiotics therapy (BIO-THREE) in patients with ulcerative colitis refractory to conventional therapy. *Scand J Gastroenterol* 2007; **42**: 1306-1311 [PMID: 17852859 DOI: 10.1080/00365520701396091]

9 **Nagashima K**, Hisada T, Sato M, Mochizuki J. Application of new primer-enzyme combinations to terminal restriction fragment length polymorphism profiling of bacterial populations in human feces. *Appl Environ Microbiol* 2003; **69**: 1251-1262 [PMID: 12571054 DOI: 10.1128/AEM.69.2.1251-1262.2003]

10 **Kibe R**, Sakamoto M, Yokota H, Ishikawa H, Aiba Y, Koga Y, Benno Y. Movement and fixation of intestinal microbiota after administration of human feces to germfree mice. *Appl Environ Microbiol* 2005; **71**: 3171-3178 [PMID: 15933018 DOI: 10.1128/AEM.71.6.3171-3178.2005]

11 **Liu WT**, Marsh TL, Cheng H, Forney LJ. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Appl Environ Microbiol* 1997; **63**: 4516-4522 [PMID: 9361437]

12 **Sakamoto M**, Hayashi H, Benno Y. Terminal restriction fragment length polymorphism analysis for human fecal microbiota and its application for analysis of complex bifidobacterial communities. *Microbiol Immunol* 2003; **47**: 133-142 [PMID: 12680716 DOI: 10.1111/j.1348-0421.2003.tb02796.x]

13 **Sepehri S**, Kotlowski R, Bernstein CN, Krause DO. Microbial diversity of inflamed and noninflamed gut biopsy tissues in inflammatory bowel disease. *Inflamm Bowel Dis* 2007; **13**: 675-683 [PMID: 17262808 DOI: 10.1002/ibd.20101]

14 **Andoh A**, Sakata S, Koizumi Y, Mitsuyama K, Fujiyama Y, Benno Y. Terminal restriction fragment length polymorphism analysis of the diversity of fecal microbiota in patients with ulcerative colitis. *Inflamm Bowel Dis* 2007; **13**: 955-962 [PMID: 17455205 DOI: 10.1002/ibd.20151]

15 **Johnson MW**, Rogers GB, Bruce KD, Lilley AK, von Herbay A, Forbes A, Ciclitira PJ, Nicholls RJ. Bacterial community diversity in cultures derived from healthy and inflamed ileal pouches after restorative proctocolectomy. *Inflamm Bowel Dis* 2009; **15**: 1803-1811 [PMID: 19637361 DOI: 10.1002/ibd.21022]

16 **Kohyama A**, Ogawa H, Funayama Y, Takahashi K, Benno Y, Nagasawa K, Tomita S, Sasaki I, Fukushima K. Bacterial population moves toward a colon-like community in the pouch after total proctocolectomy. *Surgery* 2009; **145**: 435-447 [PMID: 19303993 DOI: 10.1016/j.surg.2008.12.003]

17 **Nishikawa J**, Kudo T, Sakata S, Benno Y, Sugiyama T. Diversity of mucosa-associated microbiota in active and inactive ulcerative colitis. *Scand J Gastroenterol* 2009; **44**: 180-186 [PMID: 18825588 DOI: 10.1080/00365520802433231]

18 **Ishikawa H**, Matsumoto S, Ohashi Y, Imaoka A, Setoyama H, Umesaki Y, Tanaka R, Otani T. Beneficial effects of probiotic bifidobacterium and galacto-oligosaccharide in patients with ulcerative colitis: a randomized controlled study. *Digestion* 2011; **84**: 128-133 [PMID: 21525768 DOI: 10.1159/000322977]

19 **Wildt S**, Nordgaard I, Hansen U, Brockmann E, Rumessen JJ. A randomised double-blind placebo-controlled trial with Lactobacillus acidophilus La-5 and Bifidobacterium animalis subsp. lactis BB-12 for maintenance of remission in ulcerative colitis. *J Crohns Colitis* 2011; **5**: 115-121 [PMID: 21453880 DOI: 10.1016/j.crohns.2010.11.004]

20 **Fujimori S**, Gudis K, Mitsui K, Seo T, Yonezawa M, Tanaka S, Tatsuguchi A, Sakamoto C. A randomized controlled trial on the efficacy of synbiotic versus probiotic or prebiotic treatment to improve the quality of life in patients with ulcerative colitis. *Nutrition* 2009; **25**: 520-525 [PMID: 19201576 DOI: 10.1016/j.nut.2008.11.017]

21 **Groeger D**, O'Mahony L, Murphy EF, Bourke JF, Dinan TG, Kiely B, Shanahan F, Quigley EM. Bifidobacterium infantis 35624 modulates host inflammatory processes beyond the gut. *Gut Microbes* 2013; **4**: 325-339 [PMID: 23842110 DOI: 10.4161/gmic.25487]

22 **Andoh A**, Shimada M, Araki Y, Fujiyama Y, Bamba T. Sodium butyrate enhances complement-mediated cell injury via down-regulation of decay-accelerating factor expression in colonic cancer cells. *Cancer Immunol Immunother* 2002; **50**: 663-672 [PMID: 11862418]

23 **Andoh A**, Fujiyama Y, Hata K, Araki Y, Takaya H, Shimada M, Bamba T. Counter-regulatory effect of sodium butyrate on tumour necrosis factor-alpha (TNF-alpha)-induced complement C3 and factor B biosynthesis in human intestinal epithelial cells. *Clin Exp Immunol* 1999; **118**: 23-29 [PMID: 10540155 DOI: 10.1046/j.1365-2249.1999.01038.x]

24 **Den Hond E**, Hiele M, Evenepoel P, Peeters M, Ghoos Y, Rutgeerts P. In vivo butyrate metabolism and colonic permeability in extensive ulcerative colitis. *Gastroenterology* 1998; **115**: 584-590 [PMID: 9721155 DOI: 10.1016/S0016-5085(98)70137-4]

25 **Steinhart AH**, Hiruki T, Brzezinski A, Baker JP. Treatment of left-sided ulcerative colitis with butyrate enemas: a controlled trial. *Aliment Pharmacol Ther* 1996; **10**: 729-736 [PMID: 8899080 DOI: 10.1046/j.1365-2036.1996.d01-509.x]

26 **Roda A**, Simoni P, Magliulo M, Nanni P, Baraldini M, Roda G, Roda E. A new oral formulation for the release of sodium butyrate in the ileo-cecal region and colon. *World J Gastroenterol* 2007; **13**: 1079-1084 [PMID: 17373743 DOI: 10.3748/wjg.v13.i7.1079]

27 **Hamer HM**, Jonkers DM, Renes IB, Vanhoutvin SA, Kodde A, Troost FJ, Venema K, Brummer RJ. Butyrate enemas do not affect human colonic MUC2 and TFF3 expression. *Eur J Gastroenterol Hepatol* 2010; **22**: 1134-1140 [PMID: 20461009 DOI: 10.1097/MEG.0b013e32833a6ca0]

28 **Hamer HM**, Jonkers DM, Vanhoutvin SA, Troost FJ, Rijkers G, de Bruïne A, Bast A, Venema K, Brummer RJ. Effect of butyrate enemas on inflammation and antioxidant status in the colonic mucosa of patients with ulcerative colitis in remission. *Clin Nutr* 2010; **29**: 738-744 [PMID: 20471725 DOI: 10.1016/j.clnu.2010.04.002]

29 **Hosoe N**, Suzuki Y, Shirai K. [1-13C] sodium butyrate breath test in patients with active and quiescent ulcerative colitis by colonoscopic examination. *Dig Absorpt* 2008; **31**: 43-47

30 **Mitsui R**, Ono S, Karaki S, Kuwahara A. Neural and non-neural mediation of propionate-induced contractile responses in the rat distal colon. *Neurogastroenterol Motil* 2005; **17**: 585-594 [PMID: 16078948]

31 **Mitsui R**, Ono S, Karaki S, Kuwahara A. Propionate modulates spontaneous contractions via enteric nerves and prostaglandin release in the rat distal colon. *Jpn J Physiol* 2005; **55**: 331-338 [PMID: 16336748 DOI: 10.2170/jjphysiol.RP000205]

32 **Ono S**, Karaki S, Kuwahara A. Short-chain fatty acids decrease the frequency of spontaneous contractions of longitudinal muscle via enteric nerves in rat distal colon. *Jpn J Physiol* 2004; **54**: 483-493 [PMID: 15667672 DOI: 10.2170/jjphysiol.54.483]

33 **Maslowski KM**, Vieira AT, Ng A, Kranich J, Sierro F, Yu D, Schilter HC, Rolph MS, Mackay F, Artis D, Xavier RJ, Teixeira MM, Mackay CR. Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. *Nature* 2009; **461**: 1282-1286 [PMID: 19865172]

34 **Prantera C**, Kohn A, Campieri M, Caprilli R, Cottone M, Pallone F, Savarino V, Sturniolo GC, Vecchi M, Ardia A, Bellinvia S. Clinical trial: ulcerative colitis maintenance treatment with 5-ASA: a 1-year, randomized multicentre study comparing MMX with Asacol. *Aliment Pharmacol Ther* 2009; **30**: 908-918 [PMID: 19678813 DOI: 10.1111/j.1365-2036.2009.04117.x]

35 **Green JR**, Gibson JA, Kerr GD, Swarbrick ET, Lobo AJ,

Holdsworth CD, Crowe JP, Schofield KJ, Taylor MD. Maintenance of remission of ulcerative colitis: a comparison between balsalazide 3 g daily and mesalazine 1.2 g daily over 12 months. ABACUS Investigator group. *Aliment Pharmacol Ther* 1998; **12**: 1207-1216 [PMID: 9882028 DOI: 10.1046/j.1365-2036.1998.00427.x]

36 **Miner P**, Hanauer S, Robinson M, Schwartz J, Arora S. Safety and efficacy of controlled-release mesalamine for maintenance of remission in ulcerative colitis. Pentasa UC Maintenance Study Group. *Dig Dis Sci* 1995; **40**: 296-304 [PMID: 7851193 DOI: 10.1007/BF02065413]

37 **Courtney MG**, Nunes DP, Bergin CF, O'Driscoll M, Trimble V, Keeling PW, Weir DG. Randomised comparison of olsalazine and mesalazine in prevention of relapses in ulcerative colitis. *Lancet* 1992; **339**: 1279-1281 [PMID: 1349676 DOI: 10.1016/0140-6736(92)91601-4]

38 **Giaffer MH**, Holdsworth CD, Lennard-Jones JE, Rodrigues CA, McIntyre PB, Manjunatha S, Baron JH, Barrison IG, Polson RJ, Hoare AM. Improved maintenance of remission in ulcerative colitis by balsalazide 4 g/day compared with 2 g/day. *Aliment Pharmacol Ther* 1992; **6**: 479-485 [PMID: 1358234]

39 **Mulder CJ**, Tytgat GN, Weterman IT, Dekker W, Blok P, Schrijver M, van der Heide H. Double-blind comparison of slow-release 5-aminosalicylate and sulfasalazine in remission maintenance in ulcerative colitis. *Gastroenterology* 1988; **95**: 1449-1453 [PMID: 2903110]

40 **Paoluzi OA**, Iacopini F, Pica R, Crispino P, Marcheggiano A, Consolazio A, Rivera M, Paoluzi P. Comparison of two different daily dosages (2.4 vs. 1.2 g) of oral mesalazine in maintenance of remission in ulcerative colitis patients: 1-year follow-up study. *Aliment Pharmacol Ther* 2005; **21**: 1111-1119 [PMID: 15854173 DOI: 10.1111/j.1365-2036.2005.02458.x]

41 **Järnerot G**, Ström M, Danielsson A, Kilander A, Lööf L, Hultcrantz R, Löfberg R, Florén C, Nilsson A, Broström O. Allopurinol in addition to 5-aminosalicylic acid based drugs for the maintenance treatment of ulcerative colitis. *Aliment Pharmacol Ther* 2000; **14**: 1159-1162 [PMID: 10971232 DOI: 10.1046/j.1365-2036.2000.00821.x]

42 **Kanauchi O**, Mitsuyama K, Araki Y, Andoh A. Modification of intestinal flora in the treatment of inflammatory bowel disease. *Curr Pharm Des* 2003; **9**: 333-346 [PMID: 12570821 DOI: 10.2174/1381612033391883]

43 **Gionchetti P**, Lammers KM, Rizzello F, Campieri M. VSL#3: an analysis of basic and clinical contributions in probiotic therapeutics. *Gastroenterol Clin North Am* 2005; **34**: 499-513, ix-x [PMID: 16084310]

44 **Bibiloni R**, Fedorak RN, Tannock GW, Madsen KL, Gionchetti P, Campieri M, De Simone C, Sartor RB. VSL#3 probiotic-mixture induces remission in patients with active ulcerative colitis. *Am J Gastroenterol* 2005; **100**: 1539-1546 [PMID: 15984978 DOI: 10.1111/j.1572-0241.2005.41794.x]

45 **Mimura T**, Rizzello F, Helwig U, Poggioli G, Schreiber S, Talbot IC, Nicholls RJ, Gionchetti P, Campieri M, Kamm MA. Once daily high dose probiotic therapy (VSL#3) for maintaining remission in recurrent or refractory pouchitis. *Gut* 2004; **53**: 108-114 [PMID: 14684584 DOI: 10.1136/gut.53.1.108]

46 **Cui HH**, Chen CL, Wang JD, Yang YJ, Cun Y, Wu JB, Liu YH, Dan HL, Jian YT, Chen XQ. Effects of probiotic on intestinal mucosa of patients with ulcerative colitis. *World J Gastroenterol* 2004; **10**: 1521-1525 [PMID: 15133865]

47 **Kruis W**, Fric P, Pokrotnieks J, Lukás M, Fixa B, Kascák M, Kamm MA, Weismueller J, Beglinger C, Stolte M, Wolff C, Schulze J. Maintaining remission of ulcerative colitis with the probiotic Escherichia coli Nissle 1917 is as effective as with standard mesalazine. *Gut* 2004; **53**: 1617-1623 [PMID: 15479682 DOI: 10.1136/gut.2003.037747]

48 **Kanauchi O**, Suga T, Tochihara M, Hibi T, Naganuma M, Homma T, Asakura H, Nakano H, Takahama K, Fujiyama Y, Andoh A, Shimoyama T, Hida N, Haruma K, Koga H, Mitsuyama K, Sata M, Fukuda M, Kojima A, Bamba T. Treatment of ulcerative colitis by feeding with germinated barley foodstuff: first report of a multicenter open control trial. *J Gastroenterol* 2002; **37 Suppl 14**: 67-72 [PMID: 12572869]

49 **Bamba T**, Kanauchi O, Andoh A, Fujiyama Y. A new prebiotic from germinated barley for nutraceutical treatment of ulcerative colitis. *J Gastroenterol Hepatol* 2002; **17**: 818-824 [PMID: 12164955 DOI: 10.1046/j.1440-1746.2002.02709.x]

50 **Mitsuyama K**, Saiki T, Kanauchi O, Iwanaga T, Tomiyasu N, Nishiyama T, Tateishi H, Shirachi A, Ide M, Suzuki A, Noguchi K, Ikeda H, Toyonaga A, Sata M. Treatment of ulcerative colitis with germinated barley foodstuff feeding: a pilot study. *Aliment Pharmacol Ther* 1998; **12**: 1225-1230 [PMID: 9882030]

51 **Suzuki A**, Mitsuyama K, Koga H, Tomiyasu N, Masuda J, Takaki K, Tsuruta O, Toyonaga A, Sata M. Bifidogenic growth stimulator for the treatment of active ulcerative colitis: a pilot study. *Nutrition* 2006; **22**: 76-81 [PMID: 16226014 DOI: 10.1016/j.nut.2005.04.013]

52 **Mallon P**, McKay D, Kirk S, Gardiner K. Probiotics for induction of remission in ulcerative colitis. *Cochrane Database Syst Rev* 2007; (**4**): CD005573 [PMID: 17943867 DOI: 10.1002/14651858.CD005573.pub2]

53 **Naidoo K**, Gordon M, Fagbemi AO, Thomas AG, Akobeng AK. Probiotics for maintenance of remission in ulcerative colitis. *Cochrane Database Syst Rev* 2011; (**12**): CD007443 [PMID: 22161412 DOI: 10.1002/14651858.CD007443.pub2]

54 **Sang LX**, Chang B, Zhang WL, Wu XM, Li XH, Jiang M. Remission induction and maintenance effect of probiotics on ulcerative colitis: a meta-analysis. *World J Gastroenterol* 2010; **16**: 1908-1915 [PMID: 20397271 DOI: 10.3748/wjg.v16.i15.1908]

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ORIGINAL ARTICLE—ALIMENTARY TRACT

NUDT15 R139C-related thiopurine leukocytopenia is mediated by 6-thioguanine nucleotide-independent mechanism in Japanese patients with inflammatory bowel disease

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Abstract
Background *NUDT15* R139C (rs116855232) is a recently identified genetic factor responsible for thiopurine-induced leukocytopenia and hair loss. In this study, we investigated the association of *NUDT15* R139C with 6-thioguanine nucleotide (6-TGN) levels and thiopurine-induced leukocytopenia in Japanese patients with inflammatory bowel disease (IBD).
Methods Two hundred and sixty-four subjects (103 healthy volunteers and 161 IBD patients treated with thiopurines) were enrolled. Genotyping for *NUDT15* R139C was performed using Custom TaqMan® SNP genotyping assays.
Results The *NUDT15* C/C, C/T, and T/T genotypes were 80.7, 18.2, and 1.1 %, respectively. The allelic frequency was 10.2 %. Among 161 IBD patients, there was no significant difference in 6-TGN levels among the *NUDT15* genotypes. Forty-five patients (27.9 %) developed leukocytopenia (WBC <3000/μl), and the C/T and T/T genotypes were significantly associated with the development of leukocytopenia ($P = 1.7 \times 10^{-5}$). In these patients, 6-TGN levels were not significantly different between *NUDT15* genotypes. *NUDT15* R139C was significantly associated with early (<8 weeks) ($P = 1.03 \times 10^{-4}$) and late (>8 weeks) leukocytopenia ($P = 4.3 \times 10^{-4}$). The decrease in WBC count at 2 and 4 weeks was significantly higher in patients with the C/T or T/T genotypes as compared to the patients with the C/C genotype. All patients with the T/T genotype ($n = 2$) developed early severe hair loss and severe leukocytopenia (<1000/μl). The logistic regression analysis revealed that *NUDT15* R139C was the sole genetic factor responsible for the thiopurine-induced leukocytopenia ($P = 0.001$).
Conclusions These results suggest that *NUDT15* R139C-related thiopurine-induced leukocytopenia is mediated by a 6-TGN-independent mechanism.

Keywords NUDT15 · IBD · Azathioprine · 6-Mercaptopurine

Introduction

The thiopurine drugs azathioprine (AZA) and 6-mercaptopurine (6-MP) are widely used to maintain clinical remission of inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC) [1–3]. Despite its efficacy, thiopurine was reported to have drug-induced toxicity, such as myelotoxicity, hepatotoxicity, hair loss, pancreatitis and gastrointestinal intolerance [4, 5]. About 15–30 % of patients discontinue therapy due to side effects [6–9].

The unfortunate side-effect profile of thiopurine despite its clinical efficacy has been explained by individual thiopurine-metabolizing activity, based on the genetic polymorphism of thiopurine-metabolizing enzymes [10, 11]. AZA and 6-MP are metabolized to 6-thioguanine nucleotides (6-TGNs) [10, 11], and 6TGNs mediate the immunosuppressive properties of AZA/6-MP via the inhibition of immune cell proliferation [10]. In this process TPMT (thiopurine *S*-methyltransferase) metabolizes thiopurines to inactive molecules, and variant *TPMT* alleles

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of lower metabolizer are associated with thiopurine-induced leukocytopenia with an elevation of 6-TGN levels [12]. A SNP (single-nucleotide polymorphism) of *TPMT* A719G has been reported to be the most prevalent allele of lower thiopurine metabolize [1, 13].

Genetic polymorphism of inosine triphosphate pyrophosphatase (ITPase) was also suspected as partly responsible for thiopurine intolerance [5, 14–17]. Genetic ITPase deficiency has been reported to result in the cellular accumulation of thioinosine triphosphate (TITP), and some studies have suggested a role for *ITPase* variants in thiopurine-induced toxicity [16, 17].

Multidrug resistance protein 4 (MRP4) functions as a cellular efflux pump for 6-MP and 6-TGNs [18]. A SNP in human *MRP4* G2269A dramatically reduces MRP4 function and results in the intracellular accumulation of 6-TGNs [18]. We have previously reported that *MRP4* G2269A is associated with higher 6-TGN levels and leads to thiopurine-induced leukocytopenia in Japanese patients with IBD [19].

In addition to this genetic predisposition, a considerable number of patients who have not inherited the *TPMT* or *MRP4* variant experience thiopurine-induced leukocytopenia [1], suggesting the contribution of unknown factors responsible for inter-individual variation in thiopurine sensitivity. In 2014, Yang et al. discovered a SNP in *NUDT15* (nucleoside diphosphate–linked moiety *X*-type motif 15) (*NUDT15* R139C) that was strongly associated with thiopurine-induced leukocytopenia [odds ratio (OR) = 35.6, $P = 4.88 \times 10^{-94}$] in Korean CD patients [20]. Sensitivity and specificity of *NUDT15* R139C for thiopurine-induced early (developed within 8 weeks) leukocytopenia were 89.4 and 93.2 %, respectively. Following this study, Kakuta et al. confirmed the association of *NUDT15* R139C with early leukocytopenia in Japanese IBD patients (OR = 28.4, $P = 1.92 \times 10^{-16}$) [21]. These findings suggest that *NUDT15* R139C is a new genetic factor responsible for thiopurine-induced myelotoxicity in East Asian populations. However, these two studies did not evaluate 6TGN levels, which is quite important to understand the functional role of *NUDT15* R139C in promoting thiopurine-induced leukocytopenia.

In this study, we investigated the association of *NUDT15* R139C genotypes with thiopurine-induced leukocytopenia and 6TGN levels. Furthermore, we focused on the association between *NUDT15* R139C and decrease in WBC (white blood cell) count.

Patients and methods

Patients

We enrolled 103 healthy volunteers (female/male 46/57) and 161 IBD patients (UC $n = 89$; CD $n = 72$) attending the gastroenterology outpatient clinic at the Hospital of Shiga University of Medical Science between May 2015 and July 2015. All subjects were Japanese, and their demographic/clinical characteristics are shown in Table 1. All patients were treated with AZA/6MP. The protocol of this study was approved by the Ethics Committee of Shiga University of Medical Science. Written informed consent was obtained from all patients.

Thiopurine treatment and adverse events

The clinical data including 6-TGN levels were reviewed from medical records. The dose of 6MP was converted to an AZA equivalent dose using a conversion factor of 2.08 [22]. Thiopurine therapy was performed according to the treatment guidelines of IBD in Japan. Thiopurines were started at a dose of 0.5–1.0 mg/kg/day (as AZA dose) and adverse events were checked at 7–14 days from the initiation. If patients have no adverse events, we increased to around 1.0 mg/kg/day. The thiopurine dose and 6-TGN levels when the WBC count reached the lowest value (nadir) were used for analysis. Leukocytopenia was graded by common toxicity criteria as follows: Grade 2, 2000–3000/μl; Grade 3, 1000–2000/μl, and Grade 4, <1000/μl [23]. Severe hair loss was defined by the need to wear wigs for long period, according to the criteria described by Kakuta et al. [21].

Table 1 Characteristics of patients and healthy volunteers enrolled in this study

	UC ($n = 89$)	CD ($n = 72$)	Healthy ($n = 103$)	Total ($n = 264$)
Gender (female/male)	44/45	20/52	46/57	110/154
Age, year[a] (mean ± SEM)	37.0 ± 1.4	32.3 ± 1.2	57.7 ± 1.8	44.1 ± 0.89
5-ASA[a], *n* (%)	79 (88.8 %)	66 (91.7 %)		
Prednisolone[a], *n* (%)	67 (75.3 %)	24 (33.3 %)		
Anti-TNF drugs[a], *n* (%)	1 (1.1 %)	29 (40.3 %)		

All of the IBD patients were treated with thiopurine drugs [azathioprine (AZA) or 6-mercaptopurine (6-MP)]. The dose of 6MP was converted to an AZA equivalent dose using a conversion factor of 2.08 [22]

[a] Age, 5-ASA, prednisolone and anti-TNF drugs are the data of thiopurine start

Genotyping of *NUDT15* R139C (rs116855232), *TPMT* A719G (rs1142345), *ITPase* C94A (rs1127354) and *MRP4* G2269A (rs3765534)

Mononuclear cells were isolated from heparinized blood using a Ficoll density gradient. The genomic DNA was isolated by a DNA extraction kit purchased from QIAGEN (Valencia, CA). Genotyping for *NUDT15* R139C (rs116855232), *TPMT* A719G (rs1142345), *ITPase* C94A (rs1127354) and *MRP4* G2269A (rs3765534) was performed using Custom TaqMan® SNP genotyping assays (ID: C_154823200_10, C_19567_20, C_27465000_10 and C_27478235_20; Applied Biosystems, Inc., Foster City, CA) in accordance with information on the Applied Biosystems website (http://www.appliedbiosystems.com).

The PCR was performed according to the manufacturer's instructions provided by Applied Biosystems. The PCR thermal cycling was as follows: initial denaturing at 95 °C for 30 s followed by 40 cycles of 92 °C for 5 s and 60 °C for 20 s. Thermal cycling was performed using a LightCycler 480 system (Roche Diagnostics, Switzerland). Each 96-well plate contained 80 samples of an unknown genotype and 4 reaction mixtures containing the reagents, but no DNA (no-template control). The no-DNA control samples were necessary for the Sequence Detection System (SDS) 7700 signal processing, as outlined in the TaqMan Allelic Discrimination Guide. The genotypes were determined visually based on the dye component fluorescent emission data depicted in the *X*–*Y* scatter-plot of the SDS software.

6-TGN assay

The heparinized blood samples were first washed, and the red blood cells were hydrolyzed by acid, and extracted with phenylmercuric acetate/ethyl acetate. The 6-TGN levels were then measured by high performance liquid chromatography, as previously described [24].

Hardy–Weinberg equilibrium (HWE)

HWE analysis was performed on the research subjects by comparing the detected distribution of allele frequencies with the theoretical distribution estimated on the basis of the SNP allelic frequencies. $P > 0.05$ (Chi-squared statistics) was considered to indicate equilibrium.

Statistical analysis

The AZA dose, 6-TGN levels, and ΔWBC (ΔHb and ΔPlts) were compared using the Mann–Whitney test. The frequencies of leukocytopenia and severe hair loss for each genotype of R139C were compared by 3 × 2 Chi-square tests in univariate analyses. Logistic regression analyses were performed to identify the associations of leukocytopenia with candidate SNPs in multivariate analyses. All analyses were performed using R software version 2.14.1. $P < 0.05$ was regarded as statistically significant.

Results

Genotype frequency

Of the 264 subjects analyzed (161 IBD patients and 103 healthy volunteers), 213 subjects showed a wild type of *NUDT15* R139C (C/C) (80.7 %) (Table 2). Forty-eight subjects showed a heterozygote of *NUDT15* R139C (C/T) (18.2 %) and 3 carried a homozygote (T/T) (1.1 %) (Table 2). The allelic frequency of *NUDT15* R139C was 10.2 % (54/528). No deviation from the Hardy–Weinberg equilibrium (HWE) was detected ($P = 0.66$).

In the same cohort, 7 subjects had a heterozygote of *TPMT* A719G (2.7 %) (Table 2), and the allelic frequency of *TPMT* A719G was 1.3 % (7/528). Sixty-three subjects showed a heterozygote of *MRP4* G2269A (23.9 %) and 5 carried a homozygote (1.9 %) (Table 2). The allelic frequency of *MRP4* G2269A was 13.8 % (73/528). A heterozygote of *ITPase* C94A was detected in 55 subjects (20.8 %) and a homozygote of *ITPase* C94A was detected 6 subjects (2.3 %) (Table 2) [25]. The allelic frequency of *ITPase* C94A was 12.7 % (67/528). These results agreed with previous reports on *TPMT*, *MPR4* and *ITPase* polymorphism in the Japanese population [7, 13, 16, 19, 25].

Association of *NUDT15* genotype with 6-TGN levels and leukocytopenia

The associations of the *NUDT15* variant with 6-TGN levels and the white blood cell (WBC) count were analyzed in 161 IBD patients treated with AZA/6-MP (Table 3). These subjects consisted of 127 patients with the wild genotype (C/C), 32 patients with the C/T genotype, and 2 patients with the T/T genotype. In 161 IBD patients (including those with and without leukocytopenia), there was no significant difference in AZA dose and 6-TGN levels at WBC nadir among the *NUDT15* genotypes (Table 3), suggesting that the *NUDT15* genotype did not affect thiopurine metabolism.

Forty-five of 161 IBD patients (27.9 %) experienced leukocytopenia (Grade 2 or higher; WBC <3000/μl), and *NUDT15* variants (C/T and T/T genotype) were significantly associated with the development of leukocytopenia [$P = 1.70 \times 10^{-5}$, odds ratio (OR) 5.82, 95 % confidence

Table 2 Genotype distribution of *NUDT15, MRP4, TPMT* and *ITPase* gene

Genotype allele	Total (n = 264)	UC (n = 89)	CD (n = 72)	Healthy (n = 103)
NUDT15 R139C				
C/C (wild type)	213 (80.7 %)	69	58	86
C/T	48 (18.2 %)	18	14	16
T/T	3 (1.1 %)	2	0	1
Allele frequency	10.2 %	–	–	–
TPMT A719G				
A/A (wild type)	257 (97.3 %)	86	70	101
A/G	7 (2.7 %)	3	2	2
G/G	0	0	0	0
Allele frequency	1.30 %	–	–	–
MRP4 G2269A				
G/G (wild type)	196 (74.2 %)	67	55	74
G/A	63 (23.9 %)	20	16	27
A/A	5 (1.9 %)	2	1	2
Allele frequency	13.8 %	–	–	–
ITPA C94A				
C/C (wild type)	203 (76.9 %)	72	56	75
C/A	55 (20.8 %)	16	16	23
A/A	6 (2.3 %)	1	0	5
Allele frequency	12.7 %	–	–	–

Table 3 Association of *NUDT15* genotype with leukocytopenia and hair loss

NUDT15 genotype	C/C (n = 127)	C/T (n = 32)	T/T (n = 2)	P value
AZA dose, mg/kg/day (mean ± SEM)	0.86 ± 0.04	0.80 ± 0.07	0.75	0.27
6TGNs levels, pmol/8 × 10^8 RBC (mean ± SEM)	341.2 ± 17.7	320.5 ± 37.3	341	0.36
Leukopenia (WBC <3000), n (%)	25 (19.6 %)	18 (56 %)	2 (100 %)	1.7×10^{-5}
Early leukocytopenia (within 8 weeks), n (%)	2 (1.6 %)	2 (6.3 %)	2 (100 %)	1.03×10^{-4}
Grade 4 (WBC <1000)	0	0	2	$6.8 \times 10^{-6\#}$
Grade 3 (WBC <2000)	1	1	0	–
Grade 2 (WBC <3000)	1	1	0	–
No leukopenia	125	30	0	–
Late leukocytopenia (after 8 weeks), n (%)	23 (18.4 %)	16 (53.3 %)	–	4.3×10^{-4}
Grade 4 (WBC <1000)	0	0	–	–
Grade 3 (WBC <2000)	3	1	–	–
Grade 2 (WBC <3000)	20	15	–	–
No leukopenia	102	14	–	–
Interval to late leukocytopenia (days) (mean ± SEM)	518.9 ± 98.6	324.8 ± 95.6	–	0.06
ΔWBC at 2 weeks (/μl) (mean ± SEM)	388.6 ± 193.9	−781.1 ± 548.0	−4.550	0.021
ΔWBC at 4 weeks (/μl) (mean ± SEM)	575 ± 180.8	−2714.3 ± 532.5	−4.430	5.7×10^{-4}
Early severe hair loss, n (%)	0	0	2 (100 %)	–

ΔWBC at 2 and/or 4 weeks: Change in WBC count 2 and/or 4 weeks after thiopurine induction. The AZA dose and 6-TGNs levels when the WBC count reached the lowest value were used for analysis

WBC white blood cell, *AZA* azathioprine, *6TGNs* 6-thioguanine nucleotides

P value for early severe leukocytopenia (Grades 3 and 4)

interval (CI) 2.59–13.1] (Table 3). This result was compatible with recent reports on Koreans ($P = 5.49 \times 10^{-5}$) [20] and Japanese ($P = 1.61 \times 10^{-5}$) [21].

Next, we investigated the association of *NUDT15* R139C with early (developed within 8 weeks) and late (developed after 8 weeks) leukocytopenia. As shown in

Table 3, *NUDT15* R139C was significantly associated with the development of early leukocytopenia ($P = 1.03 \times 10^{-4}$, OR 9.33, 95 % CI 2.83–30.8), and a much stronger association was detected with the development of early severe leukocytopenia (Grades 3 and 4) ($P = 6.8 \times 10^{-6}$, OR 22.8, 95 % CI 4.25–122.6). In particular, all patients with the T/T genotype ($n = 2$) developed Grade 4 leukocytopenia (<1000/μl) within 8 weeks.

Recently, Kakuta et al. reported that the presence of *NUDT15* R139C was not related to the development of late leukocytopenia in the Japanese population. However, we detected a significant association between the presence of *NUDT15* R139C and the development of late leukocytopenia ($P = 4.3 \times 10^{-4}$, OR 4.08, 95 % CI 1.9–8.7) (Table 3). Interval to late leukocytopenia tended to be shorter in patients with the C/T genotype than those with the wild (C/C) genotype (Table 3), but statistical significance was not detected ($P = 0.06$).

ΔWBC at 2 and 4 week

To evaluate the association of *NUDT15* R139C with the decrease rate of WBC, we calculated the ΔWBC at 2 and 4 weeks. The ΔWBC at 2 weeks was calculated by the following formula: (the ΔWBC at 2 weeks) = (WBC count at 2 weeks after thiopurine initiation−WBC count at thiopurine initiation). The ΔWBC at 4 weeks was also similarly calculated. As shown in Table 3, ΔWBC at 2 and 4 weeks was significantly higher in patients with the C/T or T/T genotypes as compared to patients with the wild genotype (C/C). In patients with the wild genotype (C/C), ΔWBC values at 2 and 4 weeks were 388.6 and 575, respectively, showing no decrease in these patients. WBC count seemed to decrease much more rapidly in patients with the T/T genotype as compared with patients with the C/T genotype (statistical analysis was not possible due to small sample number). A similar tendency was observed in the rate of decrease of platelets, but the findings were not significant (Supplementary Table 1).

Early severe hair loss

All patients with the *NUDT15* T/T genotype ($n = 2$) developed early severe hair loss, but this phenomenon was not observed in patients with the wild genotype (C/C) or C/T genotype (Table 3).

AZA dose, 6-TGN levels and prednisolone dose in IBD patients with leukocytopenia

In IBD patients with leukocytopenia, AZA dose, 6-TGN levels and prednisolone dose at WBC nadir were not different between *NUDT15* genotypes (Table 4). Furthermore, there were no statistically significant differences in AZA dose and 6-TGN levels among the *NUDT15* genotypes in patients with early and late leukocytopenia (Table 4).We also evaluated the association of other candidate SNPs (*TPMT* A719G, *MRP4* G2269A, and *ITPA* C94A) with the development of leukocytopenia, but no significant association was detected (Supplementary Table 2).

Multivariate analysis

In the logistic regression analysis with *NUDT15*, *TPMT*, *MRP4*, *ITPase* variants and 6TGNs levels, the *NUDT15* variant was only genetic factor significantly contributing to the thiopurine-induced leukocytopenia ($P = 0.001$, OR 5.38, 95 % CI 1.91–15.2) (Table 5). In addition, interaction of *NUDT15* variant with *MRP4* or *ITPase* variants was not

Table 4 AZA dose and 6TGN levels in IBD patients with leukocytopenia

NUDT15 genotype	C/C	C/T	T/T	*P* value
Initial dose of AZA (mg/kg/day)	0.72 ± 0.05 ($n = 25$)	0.73 ± 0.08 ($n = 18$)	0.75 ($n = 2$)	0.37
AZA dose at WBC nadir (mg/kg/day)[a]				
Patients with leukocytopenia	0.96 ± 0.05 ($n = 25$)	0.88 ± 0.09 ($n = 18$)	0.75 ($n = 2$)	0.26
With early leukocytopenia	0.80 ($n = 2$)	0.94 ($n = 2$)	0.75 ($n = 2$)	–
With late leukocytopenia	0.97 ± 0.08 ($n = 23$)	0.88 ± 0.10 ($n = 18$)	–	0.29
6TGN levels at WBC nadir (pmol/8 × 10^8 RBCs)[a]				
Patients with leukocytopenia	370.6 ± 34.4 ($n = 25$)	367.8 ± 55.9 ($n = 16$)	341 ($n = 2$)	0.40
With early leukocytopenia	364.0 ($n = 2$)	292.0 ($n = 2$)	341 ($n = 2$)	–
With late leukocytopenia	371.3 ± 37.5 ($n = 23$)	382.9 ± 64.5 ($n = 16$)	–	0.43
Prednisolone dose at WBC nadir (mg/day)[a]	9.2 ± 1.0 ($n = 3$)	8.3 ± 1.5 ($n = 10$)	12.5 ± 7.5 ($n = 2$)	0.31

AZA dose, 6TGN levels and prednisolone dose were expressed as mean ± SEM

WBC white blood cell, *AZA* azathioprine, *6TGNs* 6-thioguanine nucleotides

[a] The AZA dose, 6-TGNs levels and prednisolone dose when the WBC count reached the lowest value (nadir) were used for analyses

Table 5 Multivariate analysis for thiopurine-induced leukopenia (WBC <3000/μl)

Variables	Odds ratio	95 % CI	*P* value
NUDT15 variant	5.38	1.91–15.2	0.001
TPMT variant	1.31	0.17–9.97	0.79
MRP4 variant	1.71	0.62–4.74	0.30
ITPA variant	0.77	0.24–2.45	0.66
6TGNs	1	1.0–1.00	0.37

CI confidence interval

observed (Supplementary Table 3). Interaction between *NUDT15* and *TPMT* variant could not be evaluated due to small number of patients with *TPMT* variant.

Discussion

In the current study, we demonstrated that *NUDT15* R139C is common in the Japanese population (allelic frequency of 10.2 %), and this finding was compatible with other recent reports of East Asians [20, 21, 26]. The genotype frequency (wild type C/C 80.7 %, heterozygote C/T 18.2 %, homozygote 1.1 %) was similar to that found in recent reports on Koreans [20] and Japanese [21].

Thiopurine-induced leukocytopenia has been mainly explained by *TPMT* variants that induce the intracellular accumulation of 6-TGNs [1, 12]. However, *TPMT* variants can be found in some patients who experience thiopurine-associated leucopenia [19, 27]. Recently, *NUDT15* R139C (rs116855232) has been identified as a novel predictor of thiopurine-induced leukocytopenia in Korean and Japanese patients with IBD [20, 21]. The GWAS (genome-wide association study) in child patients with acute lymphocytic leukemia also proved the association between *NUDT15* R139C and thiopurine sensitivity and showed that *NUDT15* R139C was found frequently in East Asians (9.8 %) and Hispanics (3.9 %) but rarely in Europeans (0.2 %) and Africans (0 %) [26]. However, none of these studies analyzed 6-TGN levels that might aid in explaining the molecular mechanism underlying the association of *NUDT15* variants with thiopurine-induced leukocytopenia. In the present study, we confirmed that *NUDT15* R139C is a strong predictor for thiopurine-induced leukocytopenia and found that *NUDT15* R139C-associated thiopurine leukocytopenia was not accompanied by an elevation of 6-TGN levels, suggesting a thiopurine metabolism-independent mechanism.

Functional changes to NUDT15 by the T to A substitution of rs116855232 remain unclear. 8-xo-7,8-Dihydroguanine (8-oxo-Gua) is the most abundant oxidized purine base [28] generated by reactive oxygen species [29]. NUDT15 hydrolyzes the 8-oxo-Gua-containing DNA precursor (8-oxo-dGTP and 8-oxo-dGDP) to the monophosphate, thereby preventing the incorporation of 8-oxo-Gua-containing nucleotides into DNA [28, 30]. NUDT15 plays an important role in accurate DNA replication as well as in the prevention of abnormal protein synthesis under oxidative stress. Yang et al. explained NUDT15 variant-related thiopurine leukocytopenia from the viewpoint of thiopurine metabolism [26]. Thio-dGTP is one of the active molecules of 6-TGNs, and due to its molecular similarity to 8-oxo-dGDP, thio-dGTP may be hydrolyzed by NUDT15 to the inactive thio-dGMP or thio-dGDP. If *NUDT15* R139C exerts loss-of-function, *NUDT15* R139C will lead to intracellular accumulation of thio-dGTP and result in extensive DNA damage and cytotoxicity. However, this hypothesis does not align with the findings in the present study that 6-TGN levels (including thio-dGTP) were not elevated in patients with *NUDT15* variants.

Rapid development of severe leukocytopenia and severe hair loss was characteristic for patients with the *NUDT15* T/T genotype, and no patients with the C/T genotype had these issues. Severe leukocytopenia and hair loss are also manifestations of strong chemotherapy, and this suggests direct injury to bone marrow- and hair follicle-stem cells. Acute bone marrow suppression is the result of the induction of apoptosis in the proliferating bone marrow stem cells, and several studies have shown that the induction of oxidative stress is primarily responsible for the loss of bone marrow- and hair follicle-stem cells [31–33]. These findings suggest that if *NUDT15* R139C induces loss-of-function, accumulation of oxidative stress and increased apoptosis in bone marrow progenitor cells might be a potential mechanism of *NUDT15* R139C-related thiopurine leukocytopenia. The thiopurine metabolism-independent mechanism as well as the functions of NUDT15 should be extensively investigated in the future.

There have been different observations in recent studies of *NUDT15* variants in East Asians. Yang et al. reported that *NUDT15* R139C was associated with both early (developed within 8 weeks) and late (after 8 weeks) leukocytopenia in the Korean population [20], but Kakuta et al. showed that *NUDT15* R139C was associated with only early leukocytopenia, but not with late leukopenia, in the Japanese population [21]. The current study showed an association of *NUDT15* R139C with early and late leukopenia in the Japanese population, and this was compatible with the findings of Yang et al. The reason for the discrepancy in late leukocytopenia might be due to different adjustments of AZA dosage based on the development of adverse events. Based on these observations, *NUDT15* R139C is considered to be a major genetic factor contributing to the heightened thiopurine sensitivity of East Asians. This is supported by the high frequency of

NUDT15 R139C [26] and low frequency of the *TPMT* variant [13] in East Asians.

In the current study, we evaluated the effects of *NUDT15* R139C not only by evaluating the frequency of leukocytopenia but also by examining the decrease in WBC count. ΔWBC indicates overall alteration of WBC count including patients whose WBC did not decrease below 3000/μl. Therefore, ΔWBC is considered to be a better marker for the evaluation of the rapid effects of *NUDT15* R139C compared to the frequency of leukocytopenia development. ΔWBC was significantly enhanced in patients with the C/T and T/T genotypes as compared to those with the wild C/C genotype. WBC count decreased in patients with the C/T and T/T genotypes after 2 and 4 weeks, but such a decrease was not observed in patients with the wild genotype. These findings clearly indicate a rapid influence of *NUDT15* R139C on hematopoietic activity.

In conclusion, we found that *NUDT15* variant-related thiopurine-induced leukopenia was not associated with 6-TGN levels, and that a decrease in the WBC count was rapidly (within 2 weeks) induced after thiopurine initiation in patients carrying *NUDT15* R139C. Our observations suggest that *NUDT15* R139C is a new genetic factor related to the increased thiopurine sensitivity/toxicity of IBD patients. The utility of routine genotyping for *NUDT15* variants before initiating thiopurine therapy should be considered to reduce the risk of thiopurine-related severe leukocytopenia and hair loss.

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Compliance with ethical standards

Conflicts of interest The authors, except Akira Andoh, disclose no conflict of interest. Akira Andoh reports speaker fees from AbbVie and Eisai Pharmaceutical.

References

1. Amin J, Huang B, Yoon J, et al. Update 2014: advances to optimize 6-mercaptopurine and azathioprine to reduce toxicity and improve efficacy in the management of IBD. Inflamm Bowel Dis. 2015;21:445–52.
2. Lee MN, Kang B, Choi SY, et al. Relationship between azathioprine dosage, 6-thioguanine nucleotide levels, and therapeutic response in pediatric patients with IBD treated with azathioprine. Inflamm Bowel Dis. 2015;21:1054–62.
3. Timmer A, McDonald JW, Tsoulis DJ, et al. Azathioprine and 6-mercaptopurine for maintenance of remission in ulcerative colitis. Cochrane Database Syst Rev. 2012;9:CD000478.
4. Gearry RB, Barclay ML, Burt MJ, et al. Thiopurine drug adverse effects in a population of New Zealand patients with inflammatory bowel disease. Pharmacoepidemiol Drug Saf. 2004;13:563–7.
5. Van Dieren JM, Hansen BE, Kuipers EJ, et al. Meta-analysis: inosine triphosphate pyrophosphatase polymorphisms and thiopurine toxicity in the treatment of inflammatory bowel disease. Aliment Pharmacol Ther. 2007;26:643–52.
6. Gisbert JP, Gomollon F. Thiopurine-induced myelotoxicity in patients with inflammatory bowel disease: a review. Am J Gastroenterol. 2008;103:1783–800.
7. Takatsu N, Matsui T, Murakami Y, et al. Adverse reactions to azathioprine cannot be predicted by thiopurine S-methyltransferase genotype in Japanese patients with inflammatory bowel disease. J Gastroenterol Hepatol. 2009;24:1258–64.
8. Lees CW, Maan AK, Hansoti B, et al. Tolerability and safety of mercaptopurine in azathioprine-intolerant patients with inflammatory bowel disease. Aliment Pharmacol Ther. 2008;27:220–7.
9. Winter JW, Gaffney D, Shapiro D, et al. Assessment of thiopurine methyltransferase enzyme activity is superior to genotype in predicting myelosuppression following azathioprine therapy in patients with inflammatory bowel disease. Aliment Pharmacol Ther. 2007;25:1069–77.
10. Derijks LJ, Gilissen LP, Engels LG, et al. Pharmacokinetics of 6-thioguanine in patients with inflammatory bowel disease. Ther Drug Monit. 2006;28:45–50.
11. Haglund S, Taipalensuu J, Peterson C, et al. IMPDH activity in thiopurine-treated patients with inflammatory bowel disease—relation to TPMT activity and metabolite concentrations. Br J Clin Pharmacol. 2007;65(1):69–77.
12. Hiratsuka M, Inoue T, Omori F, et al. Genetic analysis of thiopurine methyltransferase polymorphism in a Japanese population. Mutat Res. 2000;448:91–5.
13. Ban H, Andoh A, Tanaka A, et al. Analysis of thiopurine *S*-methyltransferase genotypes in Japanese patients with inflammatory bowel disease. Intern Med. 2008;47:1645–8.
14. Kurzawski M, Dziewanowski K, Lener A, et al. TPMT but not ITPA gene polymorphism influences the risk of azathioprine intolerance in renal transplant recipients. Eur J Clin Pharmacol. 2009;65:533–40.
15. Allorge D, Hamdan R, Broly F, et al. ITPA genotyping test does not improve detection of Crohn's disease patients at risk of azathioprine/6-mercaptopurine induced myelosuppression. Gut. 2005;54:565.
16. Uchiyama K, Nakamura M, Kubota T, et al. Thiopurine *S*-methyltransferase and inosine triphosphate pyrophosphohydrolase genes in Japanese patients with inflammatory bowel disease in whom adverse drug reactions were induced by azathioprine/6-mercaptopurine treatment. J Gastroenterol. 2009;44:197–203.
17. Zelinkova Z, Derijks LJ, Stokkers PC, et al. Inosine triphosphate pyrophosphatase and thiopurine s-methyltransferase genotypes relationship to azathioprine-induced myelosuppression. Clin Gastroenterol Hepatol. 2006;4:44–9.
18. Krishnamurthy P, Schwab M, Takenaka K, et al. Transporter-mediated protection against thiopurine-induced hematopoietic toxicity. Cancer Res. 2008;68:4983–9.
19. Ban H, Andoh A, Imaeda H, et al. The multidrug-resistance protein 4 polymorphism is a new factor accounting for thiopurine sensitivity in Japanese patients with inflammatory bowel disease. J Gastroenterol. 2010;45:1014–21.
20. Yang SK, Hong M, Baek J, et al. A common missense variant in NUDT15 confers susceptibility to thiopurine-induced leukopenia. Nat Genet. 2014;46:1017–20.
21. Kakuta Y, Naito T, Onodera M, et al. NUDT15 R139C causes thiopurine-induced early severe hair loss and leukopenia in Japanese patients with IBD. The pharmacogenomics journal. 2015;. doi:10.1038/tpj.2015.43.

22. Sandborn W, Sutherland L, Pearson D, et al. Azathioprine or 6-mercaptopurine for inducing remission of Crohn's disease. Cochrane Database Syst Rev. 2000;(2):CD000545.
23. NCI N. National Cancer Institute, common terminology criteria for adverse events v4. NIH publication, Bethesda, MD. 2009;# 09-7473.
24. Pike MG, Franklin CL, Mays DC, et al. Improved methods for determining the concentration of 6-thioguanine nucleotides and 6-methylmercaptopurine nucleotides in blood. J Chromatogr B Biomed Sci Appl. 2001;757:1–9.
25. Marinaki AM, Ansari A, Duley JA, et al. Adverse drug reactions to azathioprine therapy are associated with polymorphism in the gene encoding inosine triphosphate pyrophosphatase (ITPase). Pharmacogenetics. 2004;14:181–7.
26. Yang JJ, Landier W, Yang W, et al. Inherited NUDT15 variant is a genetic determinant of mercaptopurine intolerance in children with acute lymphoblastic leukemia. J Clin Oncol: Off J Am Soc Clin Oncol. 2015;33:1235–42.
27. Booth RA, Ansari MT, Loit E, et al. Assessment of thiopurine *S*-methyltransferase activity in patients prescribed thiopurines: a systematic review. Ann Intern Med. 2011;154:814–23 **(W-295-818)**.
28. Takagi Y, Setoyama D, Ito R, et al. Human MTH3 (NUDT18) protein hydrolyzes oxidized forms of guanosine and deoxyguanosine diphosphates: comparison with MTH1 and MTH2. J Biol Chem. 2012;287:21541–9.
29. Valko M, Leibfritz D, Moncol J, et al. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol. 2007;39:44–84.
30. McLennan AG. The Nudix hydrolase superfamily. Cell Mol Life Sci: CMLS. 2006;63:123–43.
31. Ito K, Hirao A, Arai F, et al. Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells. Nat Med. 2006;12:446–51.
32. Ito K, Hirao A, Arai F, et al. Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells. Nature. 2004;431:997–1002.
33. Zhao J, Li H, Zhou R, et al. Foxp1 Regulates the proliferation of hair follicle stem cells in response to oxidative stress during hair cycling. PLoS One. 2015;10:e0131674.