Digestive and Liver Disease 53 (2021) 841–845

Contents lists available at ScienceDirect

Digestive and Liver Disease

journal homepage: www.elsevier.com/locate/dld

Alimentary Tract

A novel gene associated with small bowel bleeding in patients taking low-dose aspirin

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ARTICLE INFO

Article history:
Received 8 March 2021
Accepted 30 April 2021
Available online 28 May 2021

Key words:
Single nucleotide polymorphism
GSTP-1
CYP4F11
Small bowel bleeding

ABSTRACT

Objective: We have previously revealed the clinical factors and genetic polymorphisms associated with gastrointestinal mucosal injury and bleeding, induced by low-dose aspirin (LDA). After performing genome-wide analysis of single nucleotide polymorphisms (SNPs) using the Drug Metabolizing Enzymes and Transporters (DMET) system among drug metabolism and transporter genes, certain SNPs were found to increase the risk for LDA-induced small bowel bleeding. The aim of this study was to identify the SNPs involved in LDA-induced small bowel bleeding.

Subjects and methods: Subjects were patients taking LDA, with small bowel bleeding diagnosed using capsule endoscopy. We investigated the clinical characteristics and the previously identified SNPs, that were examined by the DNA direct sequence method.

Results: 56 patients with bleeding and 410 controls taking LDA were enrolled. The risk factors associated with small bowel bleeding included smoking, cerebrovascular diseases, chronic renal failure, non-steroidal anti-inflammatory drug (NSAID) or anticoagulants combination, and two SNPs (CYP4F11 20043G>A (D446N) rs1060463, GSTP1 313A>G rs1695). After propensity score matching, GSTP1 rs1695 was significantly associated with small bowel bleeding.

Conclusion: The GSTP1 SNP may be a predictive marker for small bowel bleeding among patients taking LDA.

1. Introduction

Obscure gastrointestinal bleeding (OGIB) is defined as gastrointestinal (GI) bleeding of an unknown origin after a complete evaluation by esophagogastroduodenoscopy (EGD) and colonoscopy. Overt OGIB refers to clinically evident bleeding (melena, or hematochezia), and occult OGIB manifests as iron-deficiency anemia or a positive fecal occult blood test (FOBT) [1]. OGIB is reported to be responsible for 5% of all GI bleeding cases [2], and the small intestine is the leading source of OGIB, representing up to 75% of the cases [3]. Small bowel endoscopies, including video capsule endoscopy (VCE) and double-balloon endoscopy (DBE) have enabled diagnosis and have evolved to become approaches to treating OGIB during the past decade [4].

Aspirin has been known to cause gastroduodenal mucosal injury as an adverse effect, but is regarded as safe beyond the duodenum because of its rapid absorption and the lack of an enterohepatic recirculation [5]. However, previous studies using DBE and VCE indicated that aspirin caused mucosal injury in the small intestine [6–9]. High prevalence rates of mucosal breaks in the small intestine, ranging from 30% to 91%, have been reported in patients taking low-dose aspirin (LDA), although it depends on the study design [10–14]. However, the pathogenesis and the risk factors for small bowel damage induced by LDA are not well understood, making prevention difficult. The reduction of prostaglandin production in the intestinal mucosa by suppression of cyclooxygenase (COX)-1 activity, is known to be the main cause of LDA-induced mucosal injury [15]. Other COX-independent mechanisms include the toll-like receptor 4/MyD88-dependent pathway [16], enterohepatic circulation of non-steroidal anti-inflammatory drugs (NSAIDs) [17], mitochondrial damage [18], and ischemia-reperfusion injury [19].

In this era of personalized medicine, technology allows one to identify genetic risk factors in relation to the side effects of medical therapy. In a previous study investigating two single nucleotide polymorphisms (SNPs) of cyclooxygenase-1 (COX-1), which exhibit increased sensitivity to LDA and lower prostaglandin synthesis capacity, the polymorphisms lacked statistical significance in relation to an association with bleeding peptic ulcer [20]. In our previous

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https://doi.org/10.1016/j.dld.2021.04.038
1590-8658/

studies, other genetic polymorphisms such as the *SLCO1B1* 521TT genotype and the *SLCO1B1* *1b haplotype (A388G rs2306283 and T521C rs4149056) were significantly associated with LDA-induced ulcer or its complications [21–24]. Furthermore, we reported four SNPs: CYP4F11 20043GG, CYP2D6 −2178GG, CYP24A1 18948 T allele, and GSTP1*Bc.313 G allele, associated with small bowel bleeding [25]. However, the polymorphisms of cytochrome P450 2C9 (CYP2C9*2 C430T rs1799853 and CYP2C9*3 A1075C rs1057910), which is a metabolizing enzyme of NSAID, have been reported to modify the risk of NSAID-related gastroduodenal bleeding [26]. Ishihara et al. [27] reported that CYP2C9*3 SNPs (rs1057910) were significantly associated with an increased risk of small intestinal injury in NSAID users, especially with diaphragm disease. However, there are few data on the factors related to small intestinal or lower GI events among patients taking LDA. Therefore, the aim of the present study was to confirm the previous results and identify the genetic risk factors for LDA-induced small bowel bleeding.

2. Subjects and methods

2.1. Subjects

Study subjects consisted of patients with suspected bleeding from the small intestine and controls, and all subjects had at least a one-year history of consuming 100 mg of enteric-coated aspirin (Bayer Yakuhin, Ltd, Osaka, Japan). Patients who had complaints of fresh GI bleeding or exacerbated anemia with a positive fecal occult blood test had undergone abdominal ultrasonography, upper GI endoscopy, and total colonoscopy. If the patient had no identified source in the upper GI tract and colon, bleeding from the small intestine was suspected. All the patients with suspected small bowel bleeding underwent VCE within one month, and the diagnosis of LDA-induced enteropathy was made on the basis of VCE findings such as multiple erosions and/or ulcers. Outpatients taking LDA, who had no complaint of GI bleeding, no exacerbating anemia, and no source of bleeding identified by upper GI endoscopy, were enrolled as controls. Patients were excluded if they had malignant tumorous, or inflammatory or vascular lesions causing small bowel bleeding. Patients were also excluded if they had GI cancer or other malignant lesions. Demographic data were collected at entry, including age, sex, alcohol and current smoking consumption, and drug treatments including doses and internal use periods. These data were collected through interviews using structured questionnaires and from the patients' clinical records. The most evaluated medicines were continued for more than 3 years, including aspirin, and all evaluated medicines were confirmed to be unchanged from others within 2 months. The medications which had been prescribed just before endoscopy or VCE were not evaluated.

The study was approved by the Research Ethical Committee of Kawasaki Medical School, Okayama, Japan (No.3454-2) and Sakakibara Heart Institute of Okayama, Japan (No. B201907-04). The study protocol conforms to the ethical guidelines of the 1975 declaration of Helsinki, as reflected in prior approval by the institution's human research committee. Written informed consent was obtained and the study was informed by the use of the website in both hospitals (http://h.kawasaki-m.ac.jp/data/dept_022/ekigaku_s_dtl/, https://www.sakakibara-hp.com/). An opportunity to opt out was always available.

2.2. Gene polymorphisms

A peripheral blood sample was drawn to examine gene polymorphisms. The genomic DNA of the patients was extracted from 200 μL of blood sample in EDTA, using a DNA extraction kit (FAVORGEN, Ping-Tung, Taiwan). Polymerase chain reaction and direct sequencing were performed using the primers (Suppl. Table 1) to identify polymorphisms in CYP4F11 20043G>A rs1060463, CYP2D6 -2178G>A rs28360521, CYP24A1 18948C>T rs4809957, and GSTP1 313A>G rs1695. The specimens for direct sequencing were run on an applied biosystems 3130xl genetic analyzer (Applied Biosystems, Invitrogen Life Technologies, Carlsbad, CA), in accordance with the manufacturer's recommendations.

2.3. Video capsule endoscopy

PillCam SB or SB2 (Given Diagnostic Imaging System, Given Imaging Tokyo, Japan) were used for this study. Two experienced gastroenterologists blinded to the subjects' groups separately reviewed each of the procedures for small bowel injury, and identified all suspected lesions by recording the thumb-nail photographs using a rapid reader (Given Diagnostic Imaging System, Given Imaging, Tokyo). When discrepancies in the interpretation occurred, they were discussed until consensus was reached. The patients were asked to repeat the VCE if the procedure was incomplete because of a large quantity of residue.

2.4. Statistical analysis

Values are expressed as mean ± standard deviation. Mantel–Haenszel statistics was used to assess the differences in demographic and clinical characteristics. The odds ratios (ORs) and 95% confidence intervals (CIs) were obtained using Mantel–Haenszel statistics and multiple logistic regression analysis, to identify the risk or preventive factors, after adjustment for other significant factors determined by univariate analysis.

To reduce the effect of selection bias, we applied the propensity score [28], and the propensity score model was estimated using a logistic regression model, that adjusted for the characteristics and disease-related variables of patients in the control and small bowel bleeding groups. The matched variables were eight factors that likely act as confounders, namely age, sex, active smoking, ischemic heart disease, non-cardiac vascular diseases including cerebrovascular disease, chronic renal failure, taking NSAIDs and anticoagulants including warfarin, and direct oral anticoagulant (DOAC).

Differences in the genotype frequencies between the two groups, and Hardy–Weinberg equilibrium of allele frequencies at individual loci estimated by comparing the observed and expected genotype frequencies, were assessed using the chi-squared test or Fisher's exact probability test. A two-sided p value < 0.05 was considered statistically significant. All statistical calculations were performed using SPSS (version 26 for Windows, SPSS Inc., Chicago, IL).

3. Results

A total of 466 patients (315 men and 151 women; mean age, 71.0 years), including 56 patients with suspected bleeding from the small intestine (the bleeding group) were enrolled. The demographic and clinical characteristics of the patients are shown in Table 1. The significant factors associated with small bowel bleeding were active smoking (32.1% vs. 11.2%, $p < 0.001$), cerebrovascular disease (25.0% vs. 6.1%, $p < 0.001$), and chronic renal failure (10.7% vs. 1.5%, $p < 0.001$). Age, sex, active alcohol consumption, body mass index, and other underlying diseases, were not significantly different between the two groups. The percentages of patients taking NSAIDs and anticoagulants in the bleeding group were significantly higher than those in the controls (21.4% vs. 3.4%, $p < 0.001$ and 41.1% vs. 24.6%, $p = 0.009$, respectively), but the other medications, including proton pump inhibitors (PPIs), were not associated with small bowel bleeding (Table 1).

Table 1
Patient demographic and clinical characteristics.

	Controls $n=410$	Bleeding $n=56$	p
Mean Age · years	70.8	71.9	0.396[a]
Median (25%–75%)	(66.0–77.0)	(65.0–79.5)	
Over 75 years of age (%)	153 (37.4)	28 (50.0)	0.074[b]
Sex · Male (%)	274 (66.8)	41 (73.2)	0.338[b]
Active alcohol drinking (%)	130 (31.7)	13 (23.2)	0.304[b]
Active smoking (%)	46 (11.2)	18 (32.1)	<0.001[b]
BMI (SD)	23.5 (3.1)	22.9 (3.7)	0.066[c]
Ischemic heart disease (%)	305 (74.4)	35 (62.5)	0.060[b]
Cardiac arrythmias	40 (9.8)	8 (14.3)	0.296[b]
Heart valve disease	41 (10.0)	5 (8.9)	0.801[b]
Cerebrovascular disease (%)	25 (6.1)	14 (25.0)	<0.001[b]
Diabetes mellitus (%)	120 (29.3)	19 (33.9)	0.475[b]
Chronic renal failure (%)	6 (1.5)	6 (10.7)	<0.001[b]
Anticoagulants (%)	101 (24.6)	23 (41.1)	0.009[b]
P2Y12 inhibitors (%)	114 (27.8)	22 (39.3)	0.076[b]
PPIs (%)	154 (37.6)	26 (46.4)	0.201[b]
PPIs or H2-RA (%)	288 (70.2)	36 (64.3)	0.364[b]
Ca channel blockers (%)	166 (40.5)	21 (37.5)	0.669[b]
ARB/ACE-I (%)	243 (59.3)	30 (53.6)	0.417[b]
Statin (%)	222 (54.1)	30 (53.6)	0.935[b]
Nitrite (%)	115 (28.0)	13 (23.2)	0.447[b]
NSAIDs (%)	14 (3.4)	12 (21.4)	<0.001[b]

Age is the median (25th–75th interquartile ranges).
SD, standard deviation; BMI, body mass index; PPIs, proton pump inhibitors; H2-RA, Histamine H2-receptor antagonist; ARB, Angiotensin II receptor blocker; ACE-I, Angiotensin converting enzyme inhibitor; NSAIDs, non-steroidal anti-inflammatory drugs.
The p values were calculated by Mann-Whitney U analyses (a), Chi square analyses or Fisher's exact test (b), and Student's t-test (c).

Table 2
Genotype and haplotype frequencies of the candidate genes.

		Genotype	Allele frequencies	Controls Bleeding[b]	p
CYP4F11	GG	$G=0.67$	177 (43.2%)	34 (60.7%)	0.045
20043G>A(D446N)	GA	$A=0.33$	184 (44.9%)	18 (32.1%)	
rs1060463	AA	p[a]=0.66	49 (12.0%)	4 (7.1%)	
CYP2D6	GG	$G=0.53$	106 (25.9%)	19 (35.8%)	0.303
−2178G>A	GA	$A=0.47$	212 (51.7%)	24 (42.9%)	
rs28360521	AA	p[a]=0.60	92 (22.4%)	10 (18.9%)	
CYP24A1	TT	$T=0.48$	67 (16.3%)	9 (16.7%)	0.288
18948C>T	CT	$C=0.52$	210 (51.2%)	33 (61.1%)	
rs4809957	CC	p[a]=0.13	133 (32.4%)	12 (22.2%)	
GSTP1	AA	$A=0.86$	315 (76.8%)	31 (56.4%)	0.001
313A>G(I105V)	AG	$G=0.14$	88 (21.5%)	20 (36.4%)	
rs1695	GG	p[a]=0.46	7 (1.7%)	4 (7.3%)	

The p values were calculated by Chi square analyses.
a, Hardy–Weinberg equilibrium (HWE) of allele frequencies at individual loci was assessed by comparing the observed and expected genotype frequencies.
b, CYP4F11 in 56 patents, CYP2D4 in 53 patents, CYP24A1 in 54 patents, and GSTP1 in 55 patients of 56 bleeding group were successfully genotyped.

Four candidate 4 SNPs (CYP4F11, CYP2D6, CYP24A1, and GSTP1) were evaluated in all patients. CYP4F11 and GSTP1 SNPs were significantly associated with small bowel bleeding ($p=0.045$ and $p=0.001$, respectively). The frequencies of the CYP4F11 20043GG rs1060463 (60.7% vs. 43.2%, $p=0.013$) and GSTP1*Bc.313 G allele rs1695 (43.6% vs. 23.2%, $p=0.001$) were significantly higher in the bleeding subjects than in the controls (Table 2).

Active smoking (adjusted OR 4.65, 95% CI 2.17–9.97), cerebrovascular disease (5.21, 2.12–12.8), chronic renal failure (6.94, 1.55–31.1), taking NSAIDs (5.48, 1.79–16.9), co-treatment with anticoagulants (2.84, 1.41–5.72), and G allele of GSTP1*Bc.313 rs1695 (3.00, 1.50–6.00), were significantly associated with small bowel bleeding in multivariate analysis, after adjustment for significant factors in univariate analysis (Table 3). However, a significant association was not confirmed in the CYP4F11 SNP.

Propensity score matching was used to create two groups of 43 patients each. The two groups were well matched with regard to baseline characteristics (Table 4). After adjusting for propensity score, only the GSTP1 SNP was significantly associated with small bowel bleeding, and the G allele of GSTP1*Bc.313 rs1695 was significantly higher (42.9% vs. 23.2%, $p=0.015$; OR 3.28; 95% CI 1.23–8.76) in the bleeding subjects than in the controls (Suppl. Table 2).

4. Discussion

In the present study, the GSTP1 SNP was associated with small bowel bleeding in patients taking LDA, and the G allele of GSTP1*Bc.313 rs1695 was significantly higher in the bleeding subjects than in the controls. We previously performed a genome-wide analysis of 1,936 SNPs included in the Drug Metabolizing Enzymes and Transporters (DMET) system, including 17 patients with suspected small bowel bleeding, and 18 controls, and detected 27 SNPs of 23 genes. In a further validation study in a total of 437 patients, including 37 bleeding patients, 5 SNPs of 4 genes, includ-

Table 3
Association between various related factors and small bowel bleeding induced aspirin.

	Unadjusted OR (95%CI)	Adjusted OR (95%CI)
Age	1.01 (0.98–1.05)	1.01 (0.97–1.05)
Smoking	4.14 (2.17–7.89)***	4.65 (2.17–9.97)***
Cerebrovascular disease	5.00 (2.65–9.43)***	5.21 (2.12–12.8)***
Chronic renal failure	8.08 (2.51–26.0)***	6.94 (1.55–31.1)*
Anticoagulants	2.13 (1.20–3.80)*	2.84 (1.41–5.72)**
NSAIDs	7.71 (3.36–17.7)***	5.48 (1.79–16.9)**
CYP4F11 GG	2.03 (1.15–3.60)*	1.95 (0.99–3.86)
GSTP1 G allele	2.57 (1.44–4.59)**	3.00 (1.50–6.00)**

NSAIDs, non-steroidal anti-inflammatory drugs.
The unadjusted odds ratio (OR) and 95% confidence interval (CI) were obtained by Mantel-.
Haenszel statistics, and the adjusted OR and 95% CI were obtained by multiple logistic.
regression analysis after adjustment for other factors.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

Table 4
Patient background demographic and clinical characteristics: Propensity score matched analysis.

	Controls $n = 43$	Bleeding $n = 43$	p
Age (SD)	72.6 (7.5)	72.4 (10.4)	0.112[a]
Over 75 years of age (%)	20 (46.5)	22 (51.2)	0.666[b]
Sex · Male (%)	28 (65.1)	30 (69.8)	0.645[b]
Active alcohol drinking (%)	16 (37.2)	12 (27.9)	0.357[b]
Active smoking (%)	10 (23.3)	11 (25.6)	0.802[b]
BMI (SD)	23.4 (3.4)	23.2 (3.9)	0.471[a]
Ischemic heart disease (%)	30 (69.8)	31 (72.1)	0.812[b]
Cardiac arrythmias	5 (11.6)	8 (18.6)	0.366[b]
Heart valve disease	3 (7.0)	4 (9.3)	0.808[b]
Cerebrovascular disease (%)	6 (14.0)	9 (20.9)	0.394[b]
Diabetes mellitus (%)	16 (37.2)	16 (37.2)	1.000[b]
Chronic renal failure (%)	3 (7.0)	2 (4.7)	0.500[b]
Anticoagulants (%)	21 (48.8)	20 (46.5)	0.829[b]
P2Y12 inhibitors (%)	10 (23.3)	18 (41.9)	0.066[b]
PPIs (%)	10 (23.3)	17 (39.5)	0.104[b]
PPIs or H2-RA (%)	29 (67.4)	24 (55.8)	0.268[b]
Ca channel blockers (%)	23 (53.5)	15 (34.9)	0.082[b]
ARB/ACE-I (%)	24 (55.8)	24 (55.8)	1.000[b]
Statin (%)	21 (48.8)	24 (55.8)	0.517[b]
Nitrite (%)	11 (25.6)	12 (27.9)	0.808[b]
NSAIDs (%)	4 (9.3)	4 (9.3)	0.644[b]

SD, standard deviation; BMI, body mass index; PPIs, proton pump inhibitors; H2-RA, histamine H2-receptor antagonist; ARB, angiotensin II receptor blocker; ACE-I, angiotensinconverting enzyme inhibitor; NSAIDs, non-steroidal anti-inflammatory drugs.
The p values were calculated by Student's t-test (a), and Chi square analyses or Fisher's exact test (b).

ing CYP4F11 and GSTP1, were significantly associated with small bowel bleeding, although CYP2D6 (rs28360521) GG (OR 4.11, 95% CI. 1.62–10.4) was associated with small bowel bleeding after adjustment for significant factors [25]. In the present study, increasing the number of patients with bleeding, only GSTP1 313A>G rs1695 was significantly associated with both multivariate analysis and propensity score matching. This association was first reported, and the possible association of the previously identified CYP2D6 (rs28360521) could not be confirmed.

Glutathione S-transferases (GSTs) are a multigene family of enzymes that catalyze the conjugation of glutathione (GSH) to a variety of electrophilic xenobiotics, eventually forming a mercapturic acid to be excreted into the urine. Glutathione S-transferase P1 (GSTP1) is one of the members of the GST enzyme superfamily, belonging to phase II detoxification enzymes, which are also involved in the regulation of the cellular redox state through different antioxidant catalytic and noncatalytic mechanisms [29]. GSTP1 is the most prevalent in mammalian cells, as it can be expressed in all tissues and cells, except in red blood cells. [30]. It acts as an endogenous inhibitor of several signaling molecules, including c-Jun N-terminal kinase (JNK) [31], and the JNK pathway is critically involved in the pathogenesis of NSAID-induced enteropathy through the induction of mitochondria-mediated apoptosis and enterocyte death [32]. Moreover, the interaction of GSTP1 with the mitogen-activated protein kinase and NF-κB axes of regulation is also responsible for its cellular redox potential, and the regulation of kinase pathways involved in apoptosis and inflammation [33]. GSTP1 plays an important role on detoxification of reactive oxygen species (ROS) and maintenance of the cellular redox state. Oxidative stress is believed to play a crucial role in the progression of small bowel injury, because it induces modifications of cellular components such as proteins, lipids, and DNA, leading to cell dysfunction or apoptosis [34,35]. ROS production is an especially important factor in the increase of small intestinal epithelial cell permeability in the early process of small intestinal mucosal injury [36]. Moreover, Fukui et al. [37] indicated that ROS production induced by acetyl salicylic acid can specifically modify the expression of zonula occludens-1 (ZO-1) protein and induce increased cell permeability, which may ultimately cause small intestinal mucosal injury.

Almost all members of the GST family exhibit genetic polymorphisms, which can result in a complete lack or reduction of enzyme activity [38]. Two genetic variants in the GSTP1 gene have been shown to confer altered catalytic and noncatalytic activity [39]. These are the GSTP1*G allele (rs1695), encoding a protein in which amino acid isoleucine (Ile) is substituted with valine (Val) at position 105, and the GSTP1* allele (rs1138272), in which alanine (Ala) is substituted with (Val) at position 114. Simeunovic et al. [39] reported that the GSTP1 G allele (rs1695) was significantly associated with an increase in the levels of soluble intracellular adhesion molecule-1 (ICAM-1) in patients with heart failure. In an animal study, NSAID induced a rapid increase in ICAM-1 expression in blood vessels, probably because of inhibition of prostaglandin synthesis [40]. GSTP1 polymorphic variants may determine individual susceptibility to oxidative stress, inflammation, and endothelial dysfunction, and the GSTP1 G allele might decrease the antioxidant potential, providing a favorable environment for better prognosis.

In our patients, concomitant use of NSAIDs and anticoagulants was significantly associated with small bowel bleeding, as previously reported [27,41], while there was no significant association with concomitant PPI use. In a laboratory rat study by Wallace et al. [42], PPIs exacerbated NSAID-induced small bowel injury. Several clinical studies using VCE also indicated that combined LDA and PPI use could increase the risk of small bowel mucosal injury [43,44]. However, a recent study including 327 Japanese patient pairs for analysis after propensity matching, indicated no increase in risk of small bowel injuries [45] Another study also revealed that PPI did not increase the risk of symptomatic NSAID-induced small bowel injury [27]. Further prospective clinical studies on PPIs are required to confirm the exacerbation of NSAID-induced enteropathy.

There are a number of important limitations of our study. First, this was a single-center study, and the sample size of cases of small bowel bleeding was too small. FOBTs were not examined in all patients. Moreover, only four candidate SNPs detected by genome-wide SNP analysis in our preliminary study were examined without comprehensive SNP analysis. Other genes or other SNPs of GSTP1 are possibly associated with small bowel bleeding or GI mucosal injury. Moreover, the study is a retrospective case control, and selection bias should be considered when interpreting its results. To reduce the effect of selection bias and adjust

for the confounders, we applied a propensity-matched analysis in the present study, and confirmed the significant association of the GSTP1 SNP with LDA-induced small bowel bleeding. However, our study is still subject to biases from unobserved differences, and unmeasurable confounders, including clinical indication of VCE, other underlying diseases, and medications, cannot be ruled out.

In conclusion, active smoking, chronic renal failure, cerebrovascular disease, and the use of NSAIDs or anticoagulants, were associated with an increased risk of small bowel bleeding. The GSTP1*Bc.313 G allele SNP (rs1695) seems to be associated with high risk, and might be a novel predictive marker for small bowel bleeding among patients taking LDA. Further validation and extended data in a larger cohort are required.

Declaration of Competing Interest

None.

Acknowledgment

This study was supported in part by a Research Project Grant (R02G001) from Kawasaki Medical School. The authors thank Ms. Kazue Hiramatsu for her technical help.

Financial support

None.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.dld.2021.04.038.

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