Increased Gene Copy Number of *DEFA1A3* Is Associated With the Severity of Ulcerative Colitis

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INTRODUCTION: **_DEFA1A3_ encodes human neutrophil peptides (HNPs) 1–3 and has multiple copy number variations (CNVs). HNPs are associated with innate immunity. Ulcerative colitis (UC), a chronic inflammatory gastrointestinal disorder, is a life-threatening condition, and predictive markers of UC severity are needed. This study investigated the relationship between _DEFA1A3_ CNV and UC severity.**

METHODS: **This study enrolled 165 patients with UC. The relationship between _DEFA1A3_ CNV and disease severity was analyzed based on Mayo score, patient characteristics, and treatment methods. In addition, serum and stimulated neutrophil-derived HNP concentrations were also measured in patients with high and low _DEFA1A3_ CNV.**

RESULTS: **_DEFA1A3_ CNV was significantly correlated with Mayo score and white blood cell count ($R = 0.46$, $P < 0.0001$; $R = 0.29$, $P = 0.003$, respectively), and only high copy numbers of _DEFA1A3_ were independent factors for severe UC ($P < 0.001$, odds ratio: 1.88, 95% confidence interval, 1.34–2.61). The number of severe UC patients with high _DEFA1A3_ CNV was significantly greater than those with low CNV. We confirmed the associations between _DEFA1A3_ and UC severity using a validation cohort. In addition, the HNP concentration in high-copy number patients was significantly higher after neutrophil stimulation than that in low-copy number patients.**

DISCUSSION: **This study demonstrated that there is a correlation between _DEFA1A3_ copy number and severity in patients with UC. In addition, neutrophils from UC patients with higher _DEFA1A3_ CNV had high reactivity of secretion of HNPs after stimulation. _DEFA1A3_ CNV may be a novel severity marker and a potential therapeutic target for UC.**

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INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory gastrointestinal disorder (1). Although the pathogenesis of UC remains unknown, genetic and environmental factors resulting in an immune response to commensal bacteria seem to play a pivotal role in the development of UC. Acute severe UC (ASUC) is a potentially life-threatening condition (2,3). Although approximately half of ASUC patients will respond to intravenous corticosteroid therapy in the short term, about 20% must undergo early colectomy during their first hospital admission. After 1 or more episodes of severe flares, there is a 40% colectomy rate (4,5). Although some standard inflammatory biomarkers correlate well with UC severity, such as C-reactive protein (CRP) and the erythrocyte sedimentation rate (6), predictive markers for the severity of UC are still lacking and highly desirable.

One of the most important pathological hallmarks of UC is the accumulation of neutrophils into the intestinal tissue. Uncontrolled neutrophil infiltration is implicated in the pathogenesis and activity of UC (7,8). Human neutrophil peptides (HNPs), known as alpha-defensins, are secreted and released from active neutrophil granules on activation. HNPs have important immune-modulative functions, which are chemotactic for human monocytes, T cells, and immature dendritic cells (9,10). Our previous study showed that plasma and fecal HNPs in patients with active UC were higher than those in healthy subjects (11,12). It is possible that HNPs are an activity marker of UC, and their concentration may vary widely among patients and may change depending on the handling of blood cells.

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Genetic copy number variations (CNVs) are the most common mechanism to achieve structural genetic diversity, playing a key role in human health and in influencing immune responses to viral infection or autoimmune disease (13–16). The expression level of HNPs in neutrophils varies and depends on the CNV of the HNP-encoding gene *DEFA1A3*, which has an extensive range of copy numbers (17,18). *DEFA1A3* CNV may be associated with susceptibility to severe sepsis and hospital-acquired infection (19). However, *DEFA1A3* CNV and their involvement in UC development have not been well studied. Here, we investigated whether *DEFA1A3* CNVs are related to UC activity and explored HNP expression levels in UC patients depending on the degree of CNV.

METHODS

Study design, setting, and patient identification

In this study, we enrolled UC patients who were treated between 1994 and 2019 at Kagoshima University Hospital (research cohort) and Idzuro Imamura Hospital (validation cohort). Initially, the research cohort sample was examined to establish the number of CNV to discriminate the severity of the disease, and the CNV number in the validation cohort was examined without knowing the Mayo score beforehand. All patients were diagnosed with UC using established endoscopic, radiological, histological, and clinical criteria. Medical records of patients were registered in a prospectively collected inflammatory bowel disease (IBD) database from both hospitals until July 2020. The clinical rating of UC disease activity was based on the Mayo score (20). Disease activity was defined as follows: mild 3–5 points; moderate 6–10 points; and severe 11–12 points. ASUC is diagnosed according to Truelove and Witts criteria, which consist of bloody stool frequency $\geq$6 per day and at least one of the following: pulse rate $>$90 bpm, temperature $>$37.8 °C, hemoglobin $<$10.5 g/dL, and erythrocyte sedimentation rate $>$30 mm/hr (21). UC patients who underwent total colonoscopy or sigmoidoscopy were included. The Mayo endoscopic score was determined in the most severe region under colonoscopy or sigmoidoscopy observation. Exclusion criteria included patients with a history of total colectomy or colitic cancer, positive for the *Clostridioides difficile* toxin and pregnancy. The present study was approved by the Kagoshima University Hospital Institutional Review Board and performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all the patients who participated in the study.

Samples and DNA extraction

Clinical data and blood samples were obtained from the patients at the time of endoscopy (within 3 days from each other), either in an outpatient or inpatient setting. EDTA-stabilized peripheral whole blood samples were obtained from each patient, and genomic DNA was isolated using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. DNA concentration was measured by spectrophotometry, and samples were stored at −80 °C for analysis.

Droplet digital Polymerase Chain Reaction for *DEFA1A3*

The primers and probe for the *DEFA1A3* assay were Forward: 5′-CCTCTCACTGAGATTG-3′; Reverse: 5′-CCAGCCTGGATTTATAG-3′; and Probe: 5′-(FAM)CTTGTCTCCGAGCCTT-3′. Bio-Rad's ddPCR Copy Number Determination Assays (Bio-Rad, Hercules, CA) were used as reference gene.

Genomic DNA was digested with *Hae*III (New England Biolabs Japan, Tokyo, Japan) in NEB buffer 0 for 1 hour at 55 °C, followed by 20 minutes at 80 °C. A 22-μL mixture of 2 × ddPCR mastermix (Bio-Rad), forward and reverse primers for target and reference assays (900 nM each), probes for both assays (250 nM each), and 15 ng of digested genomic DNA was prepared and emulsified with Bio-Rad Automated Droplet Generator Oil in Bio-Rad Automated Droplet Generator (Bio-Rad) according to the manufacturer's instructions. The droplets automatically transferred to 96-well plates and heat-sealed with Easy Pierce foil sheets. Polymerase chain reaction was performed in a Bio-Rad C1000 thermal cycler with the following cycling parameters: 10 minutes at 95 °C (1 cycle), 30-second denaturation at 94 °C, 1-minute annealing and extension at 58 °C (40 cycles), 10 minutes

Table 1. Clinical characteristics of patients with ulcerative colitis

Research cohort	
No. of patients	105
Sex	
Male	63 (60)
Female	42 (40)
Median age, yr (range)	48.0 (11–87)
Median disease duration, yr (range)	9.2 (1.3–31.4)
Mayo score	
0–2	0
3–5	7 (6.7)
6–10	74 (70.5)
11–12	24 (22.8)
Extent of disease	
Rectum	4 (3.8)
Left-sided colitis	19 (18.1)
Pancolitis	82 (78.1)
Validation cohort	
No. of patients	60
Sex	
Male	37 (61.7)
Female	23 (38.3)
Median age, yr (range)	47.5 (18–74)
Median disease duration, yr (range)	12.7 (1.4–37.9)
Mayo score	
0–2	0
3–5	1 (1.7)
6–10	41 (68.3)
11–12	18 (30)
Extent of disease	
Rectum	1 (1.7)
Left-sided colitis	17 (28.3)
Pancolitis	42 (70)

All data are expressed as n (%).

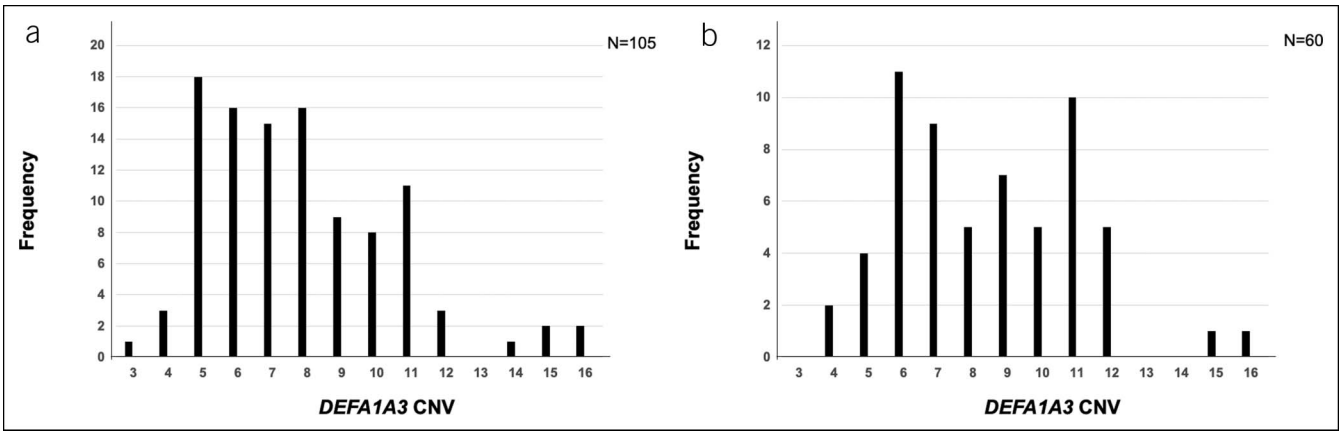


Figure 1. The frequencies of *DEFA1A3* CNV in patients with ulcerative colitis in the research cohort (**a**) and validation cohort (**b**). *DEFA1A3* CNV has a high of 16, low of 3 in the research cohort, and a high of 16, low of 4 in the validation cohort. The median of *DEFA1A3* CNV in the research and validation cohorts was 7 and 8 copies per diploid genome, respectively. CNV, copy number variation.

at 98 °C, and a hold at 12 °C. All steps had a ramp rate of 2 °C/s. Droplets were analyzed using a Bio-Rad QX200 Droplet Reader. Fluorescent data from each well were analyzed with QuantaSoft software (v1.7.4; Thermo Fisher, Waltham, MA), where copy number was calculated based on Poisson distribution (22).

Isolation of human neutrophils from peripheral blood

Whole blood samples were obtained from patients with UC. Neutrophils were isolated from EDTA-stabilized whole blood using the EasySep Direct Human Neutrophil Isolation Kit (STEMCELL, Vancouver, BC, Canada), according to the manufacturer's instructions. Cell viability was detected by trypan blue exclusion and exceeded 98%. Neutrophils were maintained in Hanks buffered saline solution supplemented with 10% heat-inactivated fetal bovine serum, sodium pyruvate, 1-mM L-glutamine, 30-μg/mL amphotericin B, 100-μg/mL streptomycin, 100-U/mL penicillin, and 100-μM 2-mercaptoethanol. Cells were counted using a hemocytometer (23).

Neutrophil activation assay

Polymorphonuclear neutrophils were adjusted to 1×10^5 cells/mL containing 900 μL of Hanks buffered saline solution, and 100-μL lipopolysaccharide (LPS; 100 ng/mL) was added to each duplicate.

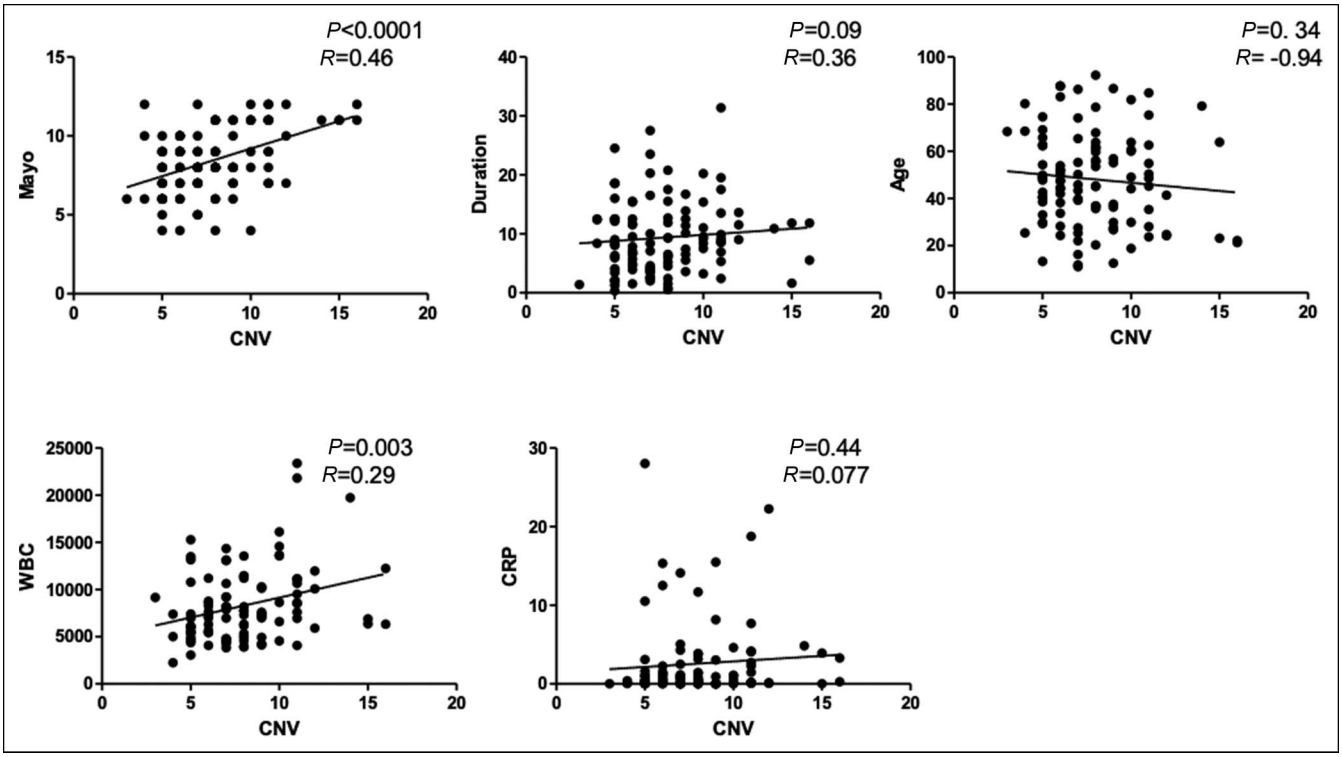


Figure 2. The correlation between the copy number of *DEFA1A3* and various clinical parameters: disease duration, age, and (measured on the most severe day in the observation period) Mayo score, WBC, and CRP. Mayo score and WBC had a significant relationship with the *DEFA1A3* copy number by Spearman rank correlation analysis ($R = 0.46$, $P < 0.001$; $R = 0.29$, $P = 0.018$; respectively). CNV, copy number variation; CRP, C-reactive protein; WBC, white blood cell.

Table 2. Clinical characteristics of the patients of the research cohort with severe and nonsevere ulcerative colitis

				Multivariate analysis		
	Severe (Mayo = 11 or 12)	Nonsevere (Mayo ≤10)	*P*	OR	95% CI	*P*
No. of patients	24	81				
Sex			0.71			
Male	14 (58.3)	49 (60.5)				
Female	10 (41.7)	32 (39.5)				
Median age, yr (range)	52.5 (21–86)	46.9 (11–87)	0.37			
Disease duration, yr (range)	8.7 (4–15.4)	9.6 (1.3–31.4)	0.91			
Extent of lesion			0.40			
Rectum	0	4 (4.9)				
Left-sided colitis	3 (12.5)	16 (19.8)				
Pancolitis	21 (87.5)	61 (75.3)				
Median *DEFA1A3* CNV	10 (4–15)	7 (4–11)	<0.001	1.88	1.34–2.61	<0.001
WBC (average ± SD), μL	9,692 ± 4,687	7,828 ± 3,527	0.065	1.01	0.99–1.01	0.93
CRP (average ± SD), mg/L	36.8 ± 53.6	22.0 ± 48.6	0.03	1.07	0.95–1.21	0.23

The severity of ulcerative colitis was defined by Mayo score. All data are expressed as n (%).
CI, confidence interval; CNV, copy number variation; CRP, C-reactive protein; OR, odds ratio; WBC, white blood cell.

The samples were incubated for 30 minutes in a 37 °C shaking water bath. To end the reaction, 1 mL of ice-cold, 0.1-M phosphate-buffered formaldehyde (10% vol/vol), pH 7.2, was added to each tube. After incubation, the supernatant was collected for enzyme-linked immunosorbent assay (ELISA) (24).

Enzyme-linked immunosorbent assay

HNP defensin peptide concentrations in patient sera and the supernatants of stimulated neutrophils were analyzed using human HNP ELISA kit (Hycult Biotech, Uden, the Netherlands) in duplicate, according to the manufacturer's instructions. The samples measured on a plate reader (Model 680 microplate reader; Bio-Rad). The concentration of the respective protein in the plasma was calculated from 450-nm absorbance readings according to a standard curve.

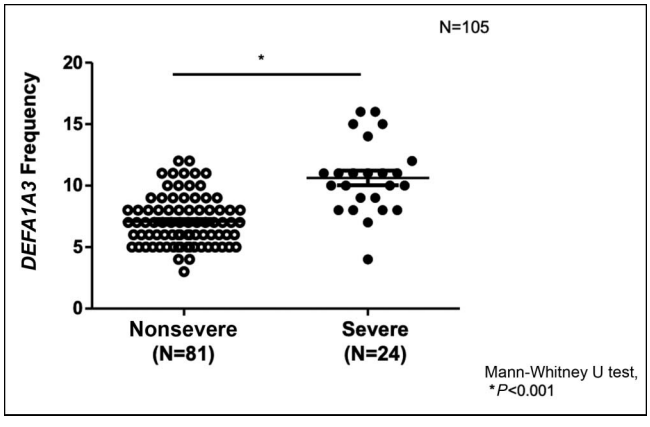


Figure 3. The frequencies of *DEFA1A3* CNV were compared between severe and nonsevere UC groups based on Mayo score. The frequencies of *DEFA1A3* CNV in the severe group were significantly higher than that of the nonsevere group ($P < 0.001$). CNV, copy number variation; UC, ulcerative colitis.

Statistical analysis

Values shown are the mean ± SD. Statistical differences were determined using the Mann-Whitney *U* test. Univariate analyses were performed using the χ^2 test for categorical variables and Spearman' rank correlation analysis. Multivariate analysis was performed using a logistic regression model with the calculation of odds ratios and 95% confidence intervals. Values of $P < 0.05$ were considered statistically significant. The discriminatory power for each putative marker was described using the receiver operating characteristic area under the curve (ROC-AUC). Statistical analyses were performed using SPSS software (SPSS, Chicago, IL).

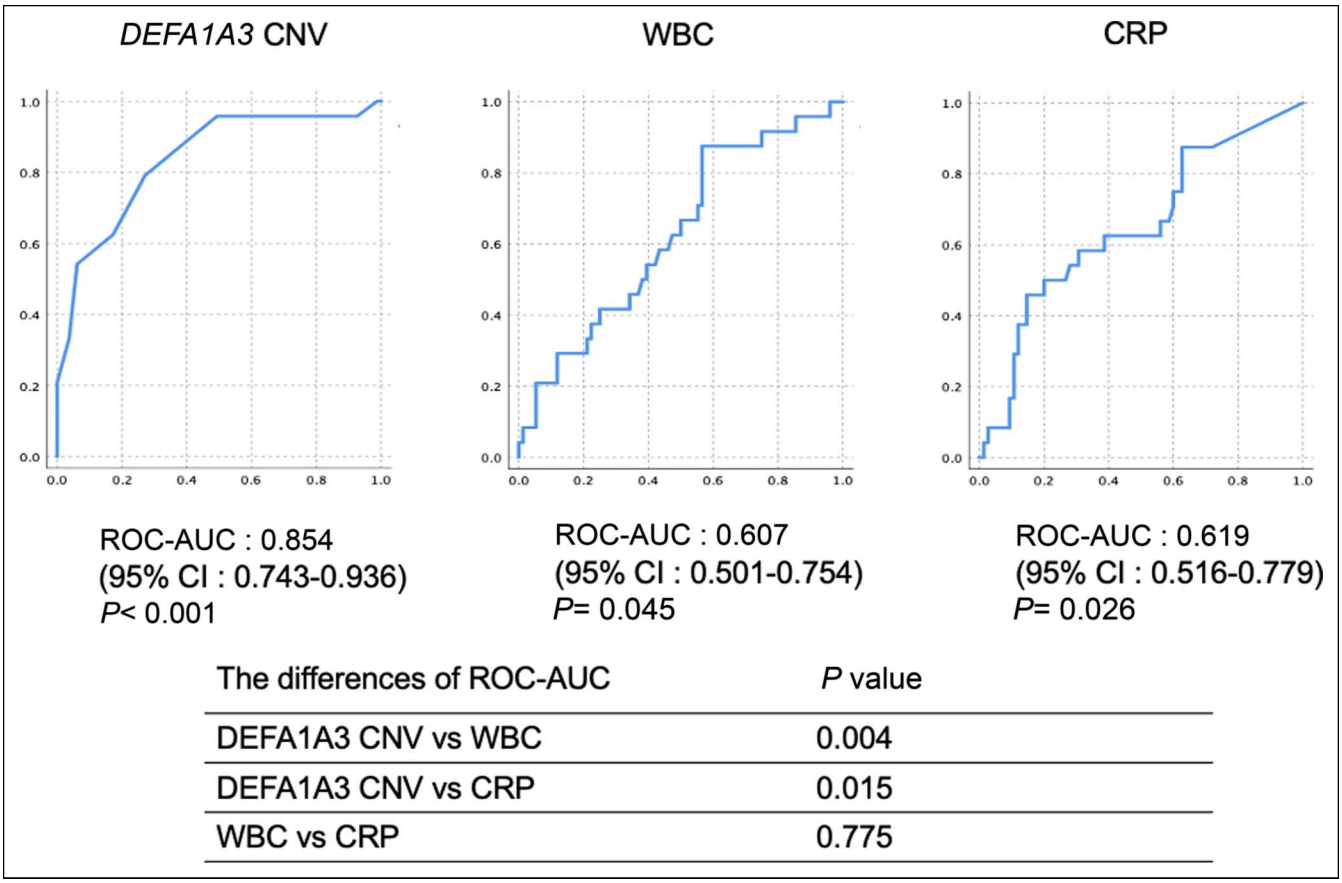
RESULTS

Patient characteristics

This study enrolled 105 patients with UC (female/male ratio: 42/63; median age at UC diagnosis: 35 years) as a research cohort. The median disease duration was 9.2 years. Among these patients, 82 had pancolitis, 19 had left-sided colitis, and 4 had proctitis. The median worst Mayo score was 8 (range, 4–12) among patients in the research cohort during observation periods (Table 1).

Distribution of *DEFA1A3* copy number in UC patients

The *DEFA1A3* copy numbers ranged from 3 to 16 per diploid genome across all UC patients of the research cohort, with a median of 7 copies (Figure 1a). We investigated the relationship between the *DEFA1A3* copy number and several parameters: age, duration of disease, Mayo score, white blood cell (WBC) count, and CRP levels on the day when the patient experienced the most severe symptoms. The Mayo score and WBC count were



The differences of ROC-AUC	*P* value
DEFA1A3 CNV vs WBC	0.004
DEFA1A3 CNV vs CRP	0.015
WBC vs CRP	0.775

Figure 4. Receiver operating characteristic area under the curves (ROC-AUCs) allow for discrimination between severe and nonsevere ulcerative colitis. ROC-AUCs of WBC, CRP, and *DEFA1A3* CNV for severe UC were 0.607 (95% CI = 0.501–0.754), 0.619 (95% CI = 0.516–0.779), and 0.854 (95% CI = 0.742–0.936) ($P = 0.045$, $P = 0.026$, and $P < 0.001$), respectively. The AUC values of *DEFA1A3* CNV vs WBC and CRP were significantly higher ($P = 0.004$ and $P = 0.015$, respectively). AUC values of WBC vs CRP were not significantly different ($P = 0.77$). CI, confidence interval; CNV, copy number variation; CRP, C-reactive protein; WBC, white blood cell.

significantly correlated with *DEFA1A3* copy number by Spearman rank correlation analysis ($R = 0.46$, $P < 0.001$; $R = 0.29$, $P = 0.003$, respectively) (Figure 2). No significant differences were observed between the *DEFA1A3* copy number and the other parameters including age, disease duration, and CRP levels ($R = -0.132$, $P = 0.21$; $R = 0.07$, $P = 0.53$; and $R = 0.021$, $P = 0.84$, respectively). In the severe Mayo score group (24 patients), the *DEFA1A3* copy number varied from 4 to 15 copies, with a median number of 10. In the nonsevere group (81 patients), the *DEFA1A3* copy number ranged from 3 to 11, with a median of 7. There were no significant differences in sex, median age, extent of UC lesion, and WBC count between the 2 groups. However, there were significant differences in the distribution of *DEFA1A3* CNV between the 2 groups (Table 2 and Figure 3).

Prediction of severity of UC based on *DEFA1A3* CNV

As shown in Table 2, univariate analysis also revealed that the severe UC group had higher *DEFA1A3* CNV, CRP, and WBC compared with the nonsevere group. Therefore, the differences in these 3 parameters in the clinical background of UC patients were subjected to multivariate analysis. The multivariate logistic regression model revealed *DEFA1A3* CNV was a significant independent predictor of severe UC. Patients with higher *DEFA1A3* CNV were associated with an increased risk of higher UC severity ($P < 0.001$, odds ratio = 1.88; 95% confidence interval = 1.34–2.61).

Diagnostic utility of *DEFA1A3* CNV to determine severity of UC

The ROC-AUCs of WBC, CRP, and *DEFA1A3* CNV for severe UC were 0.607, 0.619, and 0.854, respectively ($P = 0.045$, $P = 0.026$, and $P < 0.001$, respectively). The AUC values of *DEFA1A3* CNV vs WBC and CRP were significantly higher ($P = 0.004$ and $P = 0.015$, respectively). By contrast, no statistical differences were found between ROC-AUCs of WBC and CRP ($P = 0.77$) (Figure 4). ROC-AUC analysis revealed that a *DEFA1A3* CNV value of 9 was the optimal cutoff for discriminating between severe and nonsevere UC groups, with a sensitivity of 78.3%, specificity of 78%, positive predictive value of 50%, negative predictive value of 92.3%, and accuracy of 78.1%. Patients were then categorized into 2 groups based on copy number: High *DEFA1A3* CNV was defined as more than 9 per genome and included 36 patients. The UC severity score of patients with high *DEFA1A3* CNV was 50% (18 of 36) and that of low *DEFA1A3* CNV was 8.7% (6 of 69). This difference was statistically significant ($P = 0.001$; Table 3).

Table 3. Clinical characteristics of the patients of the research cohort in the high and low *DEFA1A3* CNV groups

	High *DEFA1A3* CNV (≥9)	Low *DEFA1A3* CNV (≤8)	*P*
No. of patients	36	69	
Sex			0.51
Male	21 (58.3)	42 (60.9)	
Female	15 (41.7)	27 (39.1)	
Median age, yr (range)	44.6 (13–86)	47.8 (11–87)	0.72
Disease duration, yr (range)	9.7 (1.6–31.4)	7.1 (1.3–27.5)	0.37
Extent of disease			0.15
Rectum	1 (2.8)	3 (4.3)	
Left-sided colitis	2 (5.6)	17 (24.6)	
Pancolitis	33 (91.6)	49 (71.1)	
Mayo score			0.001
0–10	18 (50)	63 (91.3)	
11–12	18 (50)	6 (8.7)	
Mayo endoscopic score			0.034
0–2	12 (33.3)	40 (58)	
3	24 (66.7)	29 (42)	
Immunotherapy history			
PSL	30 (83.3)	46 (44.4)	0.13
Azathioprine	21 (58.3)	28 (40.6)	0.35
TNF-α agents	15 (41.7)	29 (42)	0.84
Tacrolimus	14 (38.9)	12 (17.4)	0.11
Surgical operation for colitis	6 (16.7)	8 (11.6)	0.77

All data are expressed as n (%) except immunotherapy history which is the percentage of total patients in each group.
CNV, copy number variation; PSL, prednisolone; TNF-α, tumor necrosis factor-α.

Validation of *DEFA1A3* CNV as UC severity predictor

To determine the validity of CNV as a predictor for UC severity, we separately evaluated another patient cohort. Sixty patients with UC participated in the validation cohort. The number of women and men was 23 and 37, respectively; median age at UC diagnosis was 47.5 years, and median disease duration was 12.7 years. The clinical characteristics of the study participants are summarized in Table 1. The *DEFA1A3* copy number ranged from 3 to 16 per diploid genome across the patients of the validation cohort, with a median of 7 copies (Figure 1b). The percentage of patients with severe Mayo scores in the high *DEFA1A3* CNV group was 42.9% (12 of 28) and that of the low *DEFA1A3* CNV group was 18.6% (6 of 32) (Table 4). This difference was statistically significant.

DEFA1A3 CNV of ASUC

Because the number of patients with ASUC was small (7 in the research cohort and 3 in the validation cohort), 10 patients with ASUC were grouped together and compared the patients with non-ASUC. ASUC was diagnosed according to Truelove and Witts criteria during hospitalization. The number of women and men was 4 and 6, respectively; median age at UC diagnosis was 42.7 years (range 15–80 years) in patients with ASUC. All patients had pancolitis type and treated by predonisolone at initially. The median *DEFA1A3* copy number of ASUC patients was 10, which was the same median copy number as the severe type based on Mayo score, while the median copy number of non-ASUC patients was 7. The median *DEFA1A3* copy number of ASUC was significantly higher than that of non-ASUC. The other parameters including sex and median age were not significantly different between ASUC and non-ASUC patients.

The correlation of serum HNPs and *DEFA1A3* CNV in patients with active UC

The serum HNP concentrations in 60 patients in the validation group with UC who provided both serum and DNA samples were measured on the same day. Median serum HNP levels in this subset of patients with UC were 32.7 ng/mL (range, 8.6–124.1 ng/mL). The median serum HNP level of patients in the high *DEFA1A3* CNV group was 42.6 ng/mL (n = 28), while those in the low *DEFA1A3* group was 33.5 ng/mL (n = 43). The difference in HNP concentrations between the 2 groups was not statistically significant ($P < 0.10$). In addition, there was no correlation between the copy number of *DEFA1A3* and HNP levels ($R = 0.19$, $P = 0.10$) (Figure 5a,b).

HNPs expression from neutrophil depends on *DEFA1A3* CNV

Neutrophils were isolated from UC patients with high-copy number (n = 4), with copy numbers of 13, 11, 11, and 10, and low-copy number (n = 4), with copy numbers of 5 for all. The

Table 4. Clinical characteristics of the patients of the validation cohort in the high and low *DEFA1A3* CNV groups

	High *DEFA1A3* CNV (≥9)	Low *DEFA1A3* CNV (≤8)	*P*
No. of patients	28	32	
Sex			0.69
Male	18 (64.3)	19 (59.4)	
Female	10 (35.7)	13 (40.6)	
Median age, yr (range)	54 (23–76)	48 (18–77)	0.62
Disease duration, yr (range)	12.2 (3.2–28.5)	13.5 (1.4–37.9)	0.48
Extent of disease			0.31
Rectum	0	1 (3.1)	
Left-sided colitis	6 (21.4)	11 (34.4)	
Pancolitis	22 (78.6)	20 (62.5)	
Mayo score			0.040
0–10	16 (57.1)	26 (81.3)	
11–12	12 (42.9)	6 (18.7)	
Mayo endoscopic score			0.068
0–2	13 (46.4)	22 (68.8)	
3	15 (53.6)	10 (31.2)	
Immunotherapy history			
PSL	26 (92.9)	27 (84.4)	0.19
Azathioprine	22 (78.6)	18 (56.3)	0.067
TNF-α agents	9 (32.1)	6 (18.8)	0.23
Tacrolimus	6 (21.4)	8 (25)	0.74
Surgical operation for colitis	5 (17.9)	1 (3.1)	0.058

All data are expressed as n (%) except immunotherapy history which is the percentage of total patients in each group.
CNV, copy number variation; PSL, prednisolone, TNF-α, tumor necrosis factor-α.

concentration of HNPs in the supernatant that contained active neutrophils was evaluated using ELISA before and after LPS stimulation. Median HNP levels before stimulation in patients in the high- and low-copy number groups were 8.2 ng/mL (range, 3.8–14.3 ng/mL) and 4.8 ng/mL (range, 3.2–9.5. ng/mL), respectively. After stimulation, HNP levels of high- and low-copy number groups were 17.6 ng/mL (range, 11.2–25.2 ng/mL) and 6.9 ng/mL (range; 3.3–13.7 ng/mL), respectively (Figure 6). The HNP levels of each group were significantly increased after LPS stimulation. Notably, the HNP levels were significantly increased (approximately doubled) after stimulation compared with those in the low-copy number group.

DISCUSSION

This study demonstrated that there is a correlation between *DEFA1A3* copy number and severity in patients with UC. In addition, neutrophils from UC patients with higher CNV of *DEFA1A3* had high HNP secretion after LPS stimulation. Neutrophil infiltration of the mucosa is a common occurrence in UC and thus provides a context for the increased abundance of the neutrophil products such as HNPs in the mucosa of patients with UC. Our previous study showed that neutrophils containing high HNP levels infiltrated the large intestine, thereby intensifying inflammation. Moreover, we demonstrated that high concentrations of HNPs aggravated DSS-induced experimental colitis by elevating the levels of inflammatory cytokines (11,12,25).

HNPs differ in amino acid sequence in only the N-terminal amino acid, which is alanine for HNP-1, aspartate for HNP-3, and truncated/missing in HNP-2. These HNPs, which exhibit potent bactericidal activities against Gram-negative and -positive bacteria, are produced predominantly by neutrophils and are the most abundant neutrophil granule proteins (26,27). HNPs are directly chemotactic for T cells, macrophages, and mast cells. Furthermore, they exert effects on immunomodulatory function by inducing cytokines and chemokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β to be produced in monocytes, as well as modulating inflammatory and immune cell activation and migration, leading to damage of surrounding host tissue (28–30). In addition, HNPs attenuate the inhibitory action of dexamethasone on TNF-α production (31). Although HNPs increase production of cytokines in activated monocytes, HNPs were unable to induce TNF-α or IL-1β expression in resting monocytes (9,31–33). HNPs also induce IL-8 production from intestinal epithelial cells in a dose-dependent manner, which may stimulate additional neutrophil accumulation in the intestine (34). HNPs are suggested to play a critical role in the leukocyte-dominant proinflammatory responses in colitis. It is possible that

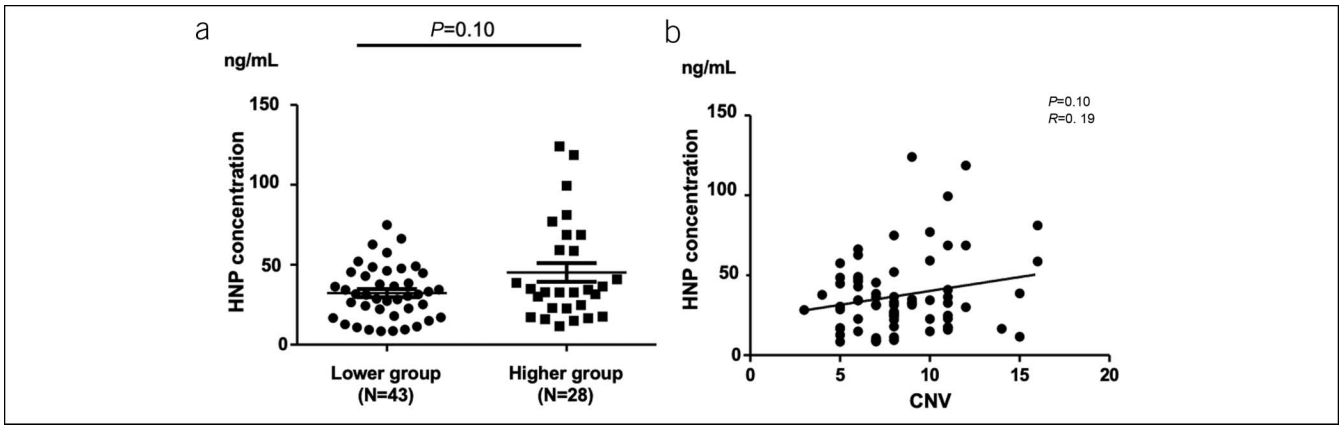


Figure 5. The serum human neutrophil peptide HNPs concentration in high and low *DEFA1A3* CNV groups (**a**). The median serum HNP level of patients in the *DEFA1A3* CNV group was 42.6 ng/mL (n = 28), while the median serum HNP level of those in the low *DEFA1A3* CNV group was 33.5 ng/mL (n = 43), $P = 0.10$ (**b**). There was no correlation between the *DEFA1A3* copy number and the serum HNP concentration ($R = 0.19$, $P = 0.10$). CNV, copy number variation; HNP, human neutrophil peptide.

higher *DEFA1A3* CNV facilitates high HNP production, resulting in severe colitis, such as ASUC.

By contrast, several recent independent reports have shown that HNPs have anti-inflammatory properties. HNP-1 reduced IL-1β and TNF-α release from activated human monocytes/macrophages (35,36). Therefore, it is possible that HNPs have a biphasic dose-dependent effect: an amelioration of inflammatory conditions at low concentrations and an aggravation at high ones.

Our previous study and other studies have shown that HNPs act on the purinergic receptor (34). The purinergic receptors are defined adenosine as binding to P1 receptors and ATP as binding to P2 receptors (37). We demonstrated that HNPs induce IL-8 production through P2Y6 through an ERK1/2-dependent mechanism in intestinal epithelial cells (34).

CNV is the most common mechanism underlying structural genetic diversity and plays a key role in human diseases. The copy numbers of the *DEFA1A3* gene in the diploid genome are between 5 and 14 in 27 healthy donors from several ethnicities, with a median number of 10 copies (17). Changes in *DEFA1A3* copy numbers have been associated with several diseases. A previous study showed that patients with more than 8 copies of *DEFA1A3* are more susceptible to severe sepsis (38,39). Patients with lower *DEFA1A3* copy numbers (fewer than 7 copies) were far more common to have hospital-acquired infections than controls (19). A lower *DEFA1A3* copy number has also been shown to increase the risk of IgA nephropathy and renal dysfunction (40). Patients with Crohn's disease, particularly with colonic involvement, have a higher *DEFA1A3* gene copy number (mean copy number 7.2 vs controls 6.7; $P < 0.001$) (41). However, another study revealed by quantitative real-time polymerase chain reaction that the median number of *DEFA1A3* CNV is 7 in patients with systemic lupus erythematosus. Moreover, no differences in *DEFA1A3* CNV were observed between systemic lupus erythematosus patients and controls (42). We suggest that *DEFA1A3* CNVs are not disease-

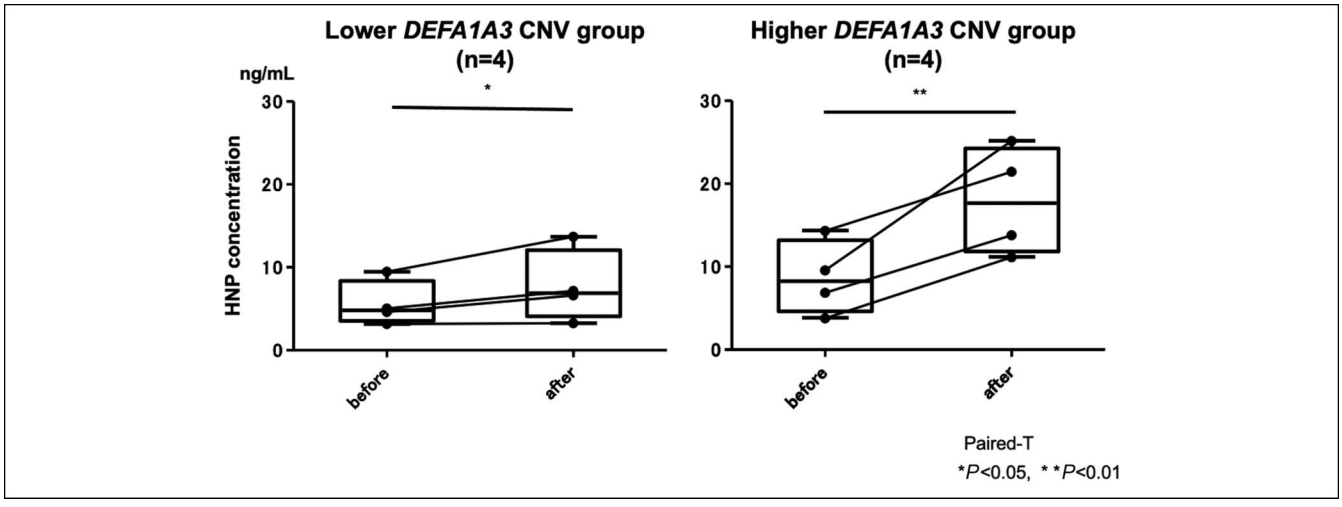


Figure 6. Neutrophils were isolated from UC patients with high *DEFA1A3* copy number (n = 4) and low copy number (n = 4). Median HNP levels before stimulation in patients with high- and low-copy numbers were 8.2 ng/mL (range, 3.8–14.3 ng/mL) and 4.8 ng/mL (range, 3.2–9.5 ng/mL), respectively. After stimulation, HNP levels in patients with high- and low-copy numbers were 17.6 ng/mL (range, 11.2–25.2 ng/mL) and 6.9 ng/mL (range, 3.3–13.7 ng/mL), respectively. CNV, copy number variation; HNP, human neutrophil peptide; UC, ulcerative colitis.

specific markers for IBD but may be used clinically as a predictive marker of severity. Furthermore, previous studies reported that the amount of HNPs expressed in neutrophils was proportional to the combined copy number of *DEFA1A3* (17,18). These findings may indicate that lower CNV decreases HNP secretion and such patients are more susceptible to bacterial infections, whereas higher CNV induces HNP secretion and is associated with cell death and inflammatory cytokine production, which may exacerbate inflammation.

Recent studies have disclosed the genetic background of IBD using novel high-throughput technologies such as genomewide association studies. Large-scale international collaborations have contributed to the identification of 200 genetic risk loci for IBD. However, the single nucleotide polymorphisms of *DEFA1A3* do not include a risk allele for IBD according to previous reports (43,44). This study found that increased copy number of the HNP-encoding gene *DEFA1A3* was a risk factor for severe UC. Neutrophil-borne HNP-1 can synergize with platelets to stimulate monocyte adhesion and enhance their recruitment, thereby participating in acute and chronic inflammation (29). Severe fulminant UC with colonic extensive ulceration causes sepsis-like symptoms (45). Patients with high HNP levels suffer from more severe sepsis because of extensive endothelial barrier dysfunction, and functional blockade of HNPs rescues mice from lethal sepsis (38). Therefore, blockade of HNPs may be a novel mechanism to prevent severe UC.

This study has several limitations. First, although this study was prospective, it enrolled a small number of patients. Therefore, we should examine a larger sample in the near future. Second, there was no correlation between copy number of *DEFA1A3* and serum HNP levels. Moreover, there was a nonsignificant trend toward increased HNP levels in the high *DEFA1A3* CNV group when compared with the low CNV group. This may be explained by differences in the serum collection timing. We found that stimulated neutrophils in the high *DEFA1A3* CNV group resulted in higher HNP secretion. It is suggested that serum HNP levels reflect intestinal HNP concentration by being released from stimulated neutrophils. Therefore, a significant positive correlation would be expected when the serum is collected at the peak of disease severity. Finally, another shortcoming was the lack of long-time follow-up. A prospective observational study is needed to determine the predictive value of *DEFA1A3* CNV. This was not possible in the current study because of the small number of patients who underwent surgical operation for colitis (Tables 3 and 4).

In conclusion, we demonstrated that there is a correlation between *DEFA1A3* copy number and clinical severity in patients with UC. In addition, neutrophils from UC patients with higher *DEFA1A3* CNV had high levels of HNP secretion after stimulation. *DEFA1A3* CNV may be a novel severity marker and a potential therapeutic target for UC.

CONFLICTS OF INTEREST

Guarantor of the article: Shuji Kanmura, MD, PhD.

Specific author contributions: S.K.: wrote the manuscript. S.K. and Y.M.: performed the experiments. A.T., Y.K., Y.S., Y.O., and H.O.: collected patient samples. H. I., K.K., S.M., F.S., S.T., S.H., and A.I.: reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

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Study Highlights

WHAT IS KNOWN

- ✓ Copy number variation is associated with disease incidence and drug susceptibility.
- ✓ Patients with acute severe ulcerative colitis are more likely to require surgery.
- ✓ There are no markers to predict the severity of ulcerative colitis.

WHAT IS NEW HERE

- ✓ There was a correlation between *DEFA1A3* copy number variation and severity in patients with ulcerative colitis.
- ✓ The concentration of encodes human neutrophil peptides secreted by neutrophils was positively associated with *DEFA1A3* copy number.

TRANSLATIONAL IMPACT

- ✓ The measurement of DEFA1A3 copy numbers can predict the severity of ulcerative colitis.

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