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Research paper

## Correlation between toll-like receptor 4 and nucleotide-binding oligomerization domain 2 (NOD2) and pathological severity in dogs with chronic gastrointestinal diseases



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## ABSTRACT

Toll-like receptor 4 (TLR4), nucleotide-binding oligomerization domain 2 (NOD2), and TNF- $\alpha$  play important roles in human inflammatory bowel diseases. The aim of this study was to elucidate the relationship between Toll-like receptor 4, NOD2, and TNF- $\alpha$  and the severity of chronic gastrointestinal diseases in dogs. We examined the expression levels of TLR4, NOD2, and TNF- $\alpha$  in the stomach, duodenum, ileum, colon, and rectum obtained from 21 dogs with chronic gastrointestinal disease, including inflammatory bowel disease, high-grade lymphoma, food responsive enteropathy, chronic pancreatitis, low-grade lymphoma, inflammatory colorectal polyp, and chronic colitis. Next, we demonstrated whether there is good correlation between the expression levels of TLR4, NOD2, and TNF- $\alpha$  and the histopathological analysis of each sample. We found that the level of TLR4 expression in the ileum of dogs with chronic gastrointestinal disease was positively associated with the histopathological severity. We also found that the level of NOD2 expression in the duodenum, stomach, and rectum was positively associated with the histopathological severity. However, there was no correlation between TNF- $\alpha$  expression in the 5 regions tested in this study and the histopathological severity. These findings indicate that TLR4 and NOD2 are remarkably associated with the severity of chronic gastrointestinal disease in dogs.

**Abbreviations:** ARE, antibiotic responsive enteropathy; CC, chronic colitis; CCECAI, canine chronic enteropathy clinical activity index; CD, Crohn's disease; CP, chronic pancreatitis; FRE, food responsive enteropathy; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HG-L, high-grade lymphoma; IBD, inflammatory bowel disease; ICP, inflammatory colorectal polyp; LG-L, low-grade lymphoma; LPS, lipopolysaccharide; LRR, leucine-rich repeat; NOD2, nucleotide-binding oligomerization domain 2; PRR, pattern recognition receptor; TLR4, toll-like receptor 4; UC, ulcerative colitis

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## 1. Introduction

The gastrointestinal tract is crucial for the immunological system and host defense, in addition to food intake, food digestion, and nutrient absorption, in humans and dogs (Azuma et al., 2008; Kinjo et al., 2017). The epithelial barrier in the intestine blocks pathogens from the lumen (Azuma et al., 2011). Inflammatory bowel disease (IBD) in dogs, as well as humans, is characterized by dysregulated intestinal inflammation and mucosal tissue damage in parts of the gastrointestinal tract (German et al., 2003; Kleinschmidt et al., 2007; Xavier and Podolsky, 2007). Some reports show that the pathology is different between humans and dogs, whereas other reports show common mucosal immune systems between humans and dogs (Allenspach et al., 2006; Cerquetella et al., 2010; German et al., 2001; Locher et al., 2001). IBD in dogs is divided into several types by histological criteria of inflammatory infiltrates, such as neutrophilic, eosinophilic, lymphocytic, plasmacytic and granulomatous infiltrates (Jergens and Simpson, 2012). In addition to dogs with IBD, dogs with food responsive enteropathy (FRE), lymphoma, and antibiotic responsive enteropathy (ARE) also develop chronic gastrointestinal disease such as vomiting, diarrhea, anorexia, bleeding, and weight loss (Dandrieux, 2016; Washabau et al., 2010). The pathological diagnosis of IBD, FRE, lymphoma, or ARE is essential to ensure the most effective treatment. To reveal the common basis of chronic gastrointestinal disease, we compared the expression levels of specific factors in the biopsy samples of dogs with chronic gastrointestinal disease using quantitative PCR. In this study, we focused on Toll-like receptor 4 (TLR4) (Kathrani et al., 2010), nucleotide-binding oligomerization domain 2 (NOD2), and TNF- $\alpha$  as specific factors. TLR4 is a transmembrane protein and member of the TLR family, which belongs to the pattern recognition receptor (PRR) family. TLR4 is best known for recognizing lipopolysaccharide (LPS), a component present in many Gram-negative bacteria. The ligands of TLR4 include several viral proteins and a variety of endogenous proteins, such as low-density lipoprotein,  $\beta$ -defensins, and heat shock protein (Brubaker et al., 2015; Schulz et al., 2005). In 2001, two groups independently identified the gene NOD2 (also called CARD15) as the first susceptibility gene for Crohn's disease (CD) (Hugot et al., 2001; Ogura et al., 2001a). NOD2 plays an important role in the immune system. NOD2 recognizes bacterial molecules such as peptidoglycans and stimulates an immune reaction. NOD2 is an intracellular PRR that recognizes molecules containing the specific structure called muramyl dipeptide that is found in certain bacteria. TNF- $\alpha$  is an important target for treating human IBD (Papamichael et al., 2017). TNF- $\alpha$  is found in higher levels in patients with IBD than in healthy people and plays a role in the development of IBD. In the present study, we investigated whether there is a good correlation between the expression levels of TLR4, NOD2, and TNF- $\alpha$ , and the histopathological analysis of each region from dogs with chronic gastrointestinal disease.

## 2. Materials and methods

### 2.1. The clinical cases

This study included 21 cases of dogs with chronic gastrointestinal signs (e.g., vomiting, diarrhea, anorexia, weight loss) for over 3 weeks. The clinical cases were referred to the Veterinary Medical Center of Osaka Prefecture University Graduate School of Life and Environmental Sciences between 2014 October and 2016 October. A diagnostic evaluation was performed in all dogs, including a complete blood count, serum biochemistry profile (trypsin-like immunoreactivity and pancreatic-lipase immunoreactivity), urinalysis, fecal examination for parasitology and bacteriology, X-ray examination, abdominal ultrasound examination, and endoscopic examination to exclude any disease other than gastrointestinal disorders. Gastrointestinal biopsy was performed in all dogs during the endoscopic examination for the histopathological analysis to detect neoplastic diseases and mucosal

inflammatory infiltration, irrespective of the diagnosis. Mucosal biopsy specimens were obtained from stomach, duodenum, ileum, colon, and rectum. Diagnostic workup for FRE and ARE was undertaken in all cases except those with neoplastic diseases and chronic pancreatitis (CP). FRE was defined as complete remission of clinical signs within 2–4 weeks under the elimination diet-test. If the patient did not respond to the initial diet, an alternative diet was tested. Although the same diet was not used in all cases, at least two kinds of hypoallergenic diet or novel antigen diet were used. A complete clinical response to therapeutic trial with metronidazole (10 to 15 mg/kg, p.o., twice daily for 2 weeks) defined the diagnosis of ARE. A diagnosis of IBD was based on clinical signs (e.g., vomiting, diarrhea, anorexia, weight loss) for over 3 weeks, exclusion of other causes of chronic gastrointestinal tract signs, and detection of inflammatory infiltration in histopathological examination of gastrointestinal biopsy samples. In severe cases with hypoalbuminemia and anemia, after excluding neoplastic diseases and any diseases other than the gastrointestinal tract, corticosteroids were used concurrently with hypoallergenic diet or novel antigen diet and metronidazole as indicated by clinical judgement. After the clinical signs were controlled, patients who relapsed during corticosteroid tapering were clinically diagnosed as IBD. All cases were scored for severity according to the canine chronic enteropathy clinical activity index (CCECAI) (Allenspach et al., 2007). The collection and analysis of all specimens obtained from dogs with chronic gastrointestinal disease were approved by the ethics committee of Veterinary Medical Center of Osaka Prefecture University, and informed consent was obtained from all owners of dogs participating in the study.

### 2.2. Diagnosis and histopathological analysis

In all cases, a biopsy was submitted for microscopic evaluation. All biopsy samples were fixed in 10% neutral buffered formalin, embedded in paraffin, and routinely stained with HE. Definitive diagnosis was based on histopathologic evidence by two pathologists. In addition, histopathological analysis was graded according to the guideline of the World Small Animal Veterinary Association international gastrointestinal standardization group (Day et al., 2008) with minor modifications as follows: 0, normal; 1, mild; 2, mild-moderate; 3, moderate; 4, moderate-severe; 5, severe.

### 2.3. RNA isolation and quantitative real-time PCR

A portion of the biopsy sample was immediately stored in RNAlater Solution (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA was isolated as previously described (Azuma et al., 2010). RNA was used to synthesize complementary cDNA using Superscript Reverse Transcriptase (Roche, Madison, WI, USA). The primers used for the amplification are described in Table 1. mRNA expression levels were quantified using real-time PCR analysis based on the intercalation of SYBR Green (Toyobo, Osaka, Japan). The amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an

**Table 1**  
Sequences of the primers used in quantitative PCR.

Target	5'-3'	
TLR4	F	C AAAATCCCAACAACATCC
	R	TGGTTTAGGCCCTGATATGC
NOD2	F	AGACCGAGGCATCTGTAAGC
	R	AGCCTCAAAGCCAGGAAGTT
TNF- $\alpha$	F	ACCACACTCTCTGCCTGCT
	R	CTGGTTGTCTGTGAGCTCCA
GAPDH	F	GGAGAAAGCTGCCAAATATG
	R	ACCAGAAATGAGCTTGACA

Forward (F) and reverse (R) sequences are listed (5'-3') for canine TLR4, canine NOD2, canine TNF- $\alpha$ , and canine GAPDH.

**Table 2**  
Breeds and numbers of dogs.

Breed	Number
Miniature Dachshund	3
Shiba	3
Chihuahua	3
Maltese Terrier	2
Miniature Schnauzer	2
Shih Tzu	1
Siberian Huskytab	1
Jack Russell Terrier	1
French Bulldog	1
Border Collie	1
Miniature Pinscher	1
Rottweiler	1
MIX	1
Total	21

endogenous control to account for the differences in the amount and quality of RNA added to each reaction.

#### 2.4. Statistical analysis

The correlation between the level of mRNA expression and the grade of histopathological analysis was evaluated by Spearman's rank correlation coefficient. The level of mRNA expression was statistically analyzed using Welch's *t*-test among IBD, high-grade lymphoma (HG-L), and FRE. Differences with *P* values of less than 0.05 were considered significant.

### 3. Results

#### 3.1. Cases of dogs

The breeds and numbers of the dogs enrolled in this study are shown in Table 2. Twenty-one dogs with chronic gastrointestinal signs were evaluated for definitive diagnosis. All dogs used in this study had evidence of inflammation within the mucosa. Definitive diagnoses were IBD (*n* = 9), HG-L (*n* = 4), FRE (*n* = 3), CP (*n* = 2), low-grade lymphoma (LG-L) (*n* = 1), inflammatory colorectal polyp (ICP) (*n* = 1), and chronic colitis (CC) (*n* = 1). In the histopathological examination results of this CC patient, severe mixed cell enteritis and ulcerative lesions were observed in the rectum. Mild-to-moderate lymphoplasmacytic enteritis were observed in the colon. The case dog was clinically diagnosed as CC because the dog showed complete remission of clinical signs after treatment with low fat diet and salazosulfapyridine. Group compositions in terms of age, gender, body weight, and CCECAI are shown in Table 3.

**Table 3**  
Details of cases of dogs.

Diagnosis	IBD	HG-L	FRE	CP	LG-L	ICP	CC
Number	9	4	3	2	1	1	1
Age (years; median [range])	8.0 [3.0-12.0]	8.5 [7.0-11.0]	9.0 [1.0-11.0]	7.0 [5.0-9.0]	9.0	9.0	5.0
Gender (male/female)	4/5	2/2	2/1	1/1	1/0	0/1	0/1
Weight (kg; median [range])	4.7 [1.8-43.0]	7.1 [3.3-10.0]	5.2 [4.2-20.4]	8.3 [7.3-9.2]	2.4	5.5	6.3
CCECAI (median [range])	11.0 [5.0-14.0]	14.0 [11.0-16.0]	6.0 [1.0-7.0]	3.0 [1.0-5.0]	16.0	3.0	1.0

#### 3.2. Correlations between the histopathological severity and the expression of TLR4, NOD2, and TNF- $\alpha$

Using Spearman's rank correlation coefficient, we analyzed whether the level of TLR4, NOD2, and TNF- $\alpha$  mRNA expression in the biopsy tissues correlated with the histopathological severity in each region of dogs with chronic gastrointestinal disease. Significant positive correlation was detected between the level of TLR4 expression and the histopathological severity in the ileum ( $r_s = 0.47$ ) (Fig. 1). However, there was no significant correlation in the other 4 regions. Similar to TLR4, significant positive correlations were detected between the level of NOD2 expression and the histopathological severity in the duodenum ( $r_s = 0.44$ ), the stomach ( $r_s = 0.47$ ) and the rectum ( $r_s = 0.78$ ) (Fig. 1). However, there was no significant correlation in the other 2 regions. Unlike NOD2 and TLR4, there were no significant correlations between the level of TNF- $\alpha$  expression and the histopathological severity in any of the 5 regions tested (Fig. 1).

#### 3.3. Comparisons of the mRNA expression level among IBD, HG-L, and FRE

Next, we compared the region-specific levels of TLR4, NOD2 and TNF- $\alpha$  mRNA expression among dogs with IBD, HG-L, and FRE. The TLR4 expression levels in the ileum obtained from dogs with FRE were markedly but not significantly lower than those with IBD ( $P = 0.05602$ ) (Fig. 2A). In contrast, the level of TLR4 expression in the ileum was not significantly different between HG-L and FRE. Unlike the ileum, however, the level of TLR4 expression in the other 3 regions was not significantly different among IBD, HG-L, and FRE.

In the ileum, the level of NOD2 expression was significantly lower in dogs with FRE than in dogs with IBD ( $P < 0.05$ ) and HG-L ( $P < 0.01$ ) (Fig. 2B). In the stomach, the level of NOD2 expression was markedly, but not significantly, lower in dogs with FRE than in dogs with IBD ( $P = 0.05329$ ). In contrast, the level of NOD2 expression in the stomach was not significantly different between dogs with HG-L and FRE. Unlike the ileum and stomach, however, the level of NOD2 expression in the duodenum and colon was not significantly different among dogs with IBD, HG-L, and FRE.

Unlike TLR4 and NOD2, the level of TNF- $\alpha$  expression in the 4 regions tested was not significantly different among dogs with IBD, HG-L, and FRE (Fig. 2C).

### 4. Discussion

We observed that TLR4, NOD2, and TNF- $\alpha$  showed different patterns of correlation between expression level and histopathological severity. Interestingly, the level of TLR4 expression in the ileum of dogs with chronic gastrointestinal disease was positively associated with the histopathological severity. Importantly, the level of NOD2 expression in the duodenum, stomach, and rectum was positively associated with the histopathological severity. In contrast, there was no correlation between TNF- $\alpha$  and the histopathological severity in the 5 regions tested in this study. Clinically, the duodenum and the ileum are more important than the stomach, the colon, and the rectum in chronic gastrointestinal disease. We introduce these previous studies to discuss our results.

TLRs are PRRs and are key immune sensors of microbiota in the gut (Schirbel et al., 2019). In addition, TLRs not only control innate immunity but also critically regulate adaptive immunity, such as T cell activation. The balance between regulatory T cells and effector T cells is disturbed in human patients with IBD and mice with IBD (Shi et al., 2019; Zhu et al., 2017; Zhang et al., 2018). TLRs are expressed in both the intestinal epithelial cells and stromal tissue cells of the gastrointestinal tract. In particular, TLR4 is detected in the small and large intestines of mice and humans (Moossavi and Rezaei, 2013). Considering that the expression of TLR4 mRNA is upregulated in human patients with active ulcerative colitis (UC), TLR4 might be a participant

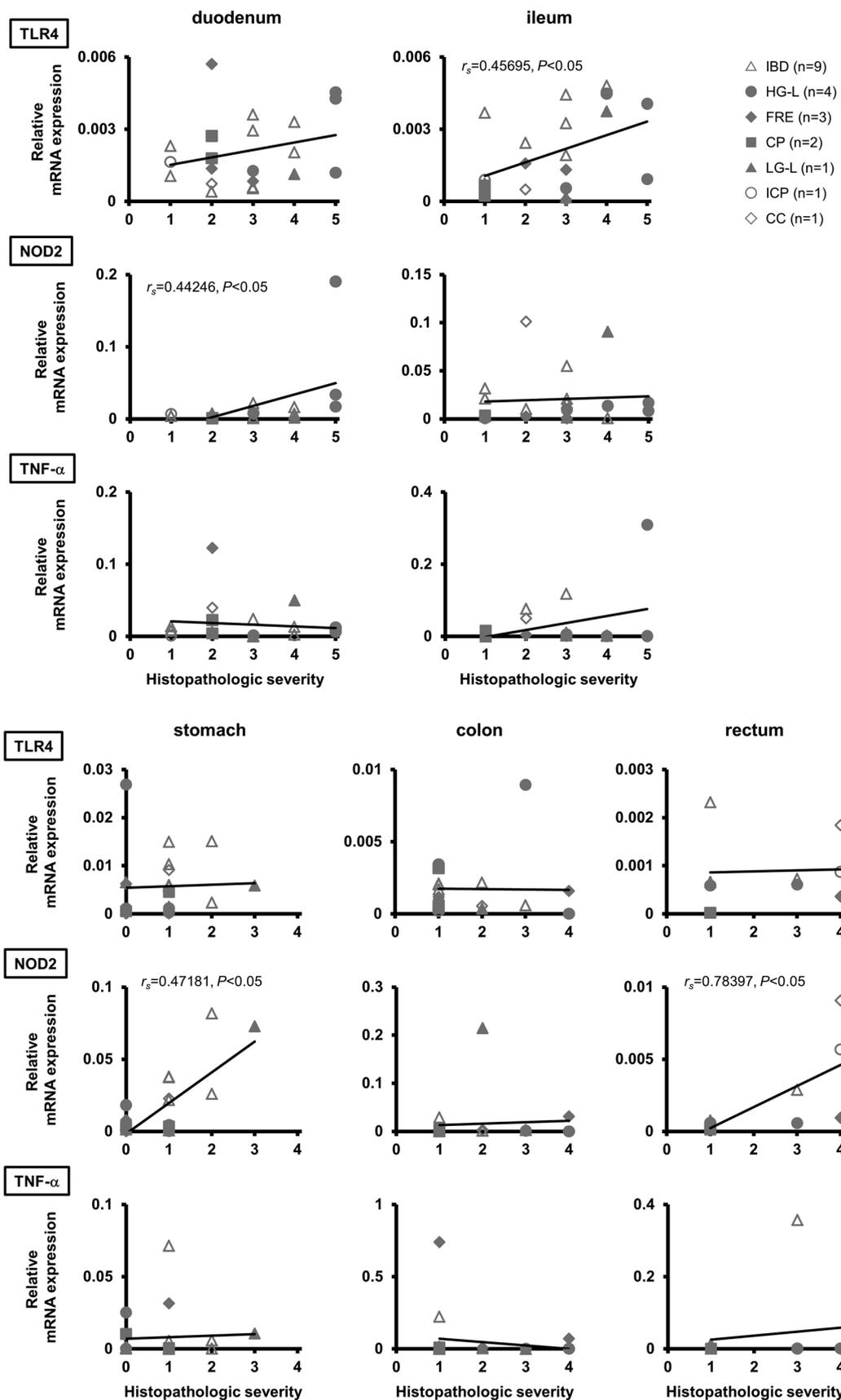


Fig. 1. Analysis of the correlations between the level of mRNA expression and the histopathological severity in dogs with chronic gastrointestinal disease by Spearman's rank correlation coefficient. Duodenum, stomach, and colon (n = 21). Ileum (n = 20). Rectum (n = 9).

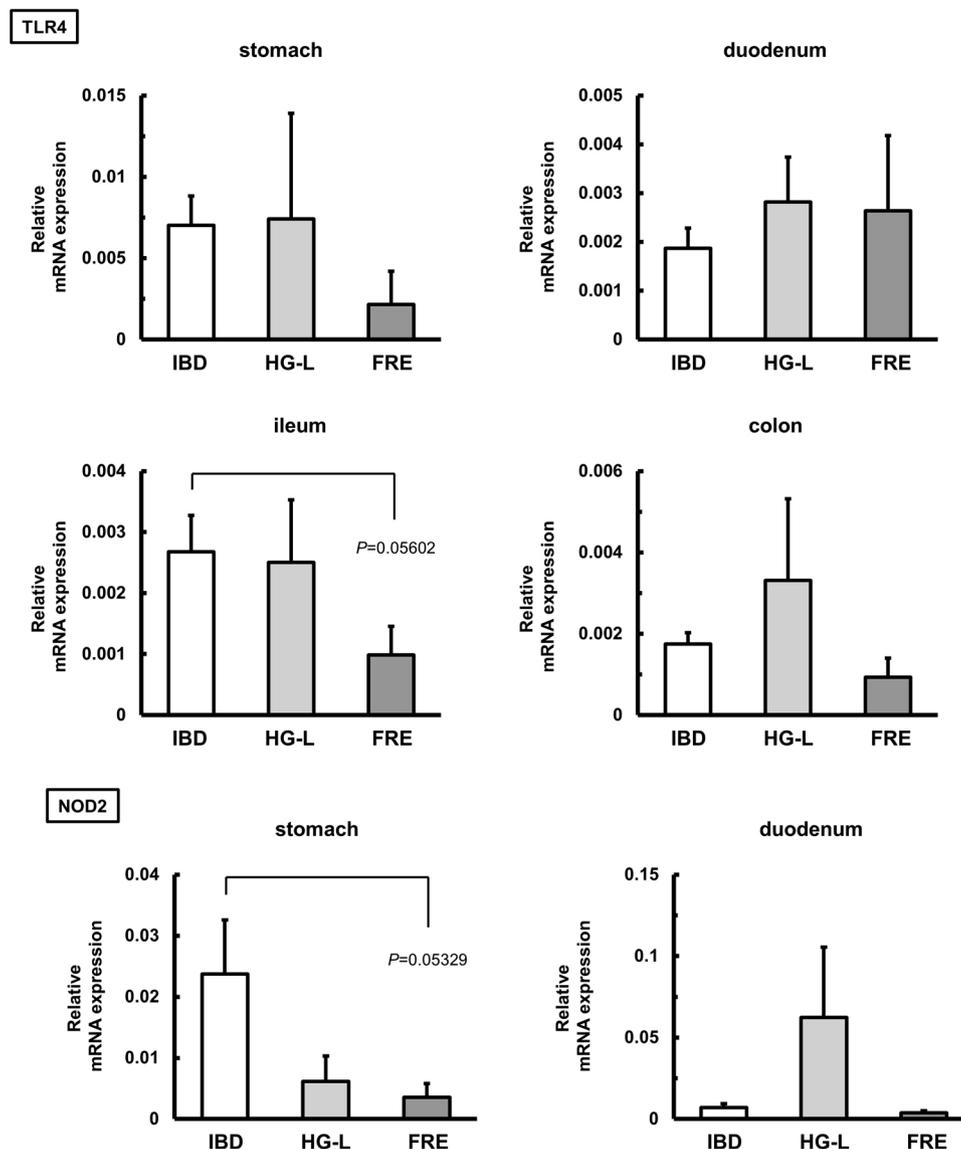


Fig. 2. mRNA expression among dogs with IBD, HG-L, and FRE. The level of mRNA expression of TLR4 (A), NOD2 (B), and TNF- $\alpha$  (C) in each gastrointestinal region of dogs with IBD (n = 9), HG-L (n = 4), and FRE (n = 3).

in UC disease development. The TLR4 signals were significantly greater in the inflamed colon compared with non-inflamed colon of miniature dachshunds with ICP and healthy beagles (Yokoyama et al., 2017a,b). In addition, levels of TLR4 were significantly upregulated in the ICP in the colon of miniature dachshunds relative to controls (Igarashi et al., 2014). Moreover, TLR4 expression was increased in the duodenum, ileum, and colon of German shepherd dogs with chronic enteropathies (Allenspach et al., 2010). TLR ligands, including LPS, upregulated the expression of TLR4 in primary colonic epithelial cells (Swerdlow et al., 2006) and canine macrophage cell line DH82 cells (Fujimoto et al., 2012). In contrast, TLR4 mRNA expression was similar in the duodenum of dogs with IBD and control cases (McMahon et al., 2010). These data suggest that TLR4 may be involved in dogs with chronic gastrointestinal diseases, similar to human IBD. We have to discuss the interesting and unanswered question of why there is only a positive correlation in the ileum. It was reported that basal TLR6 expression levels are different between the proximal and distal colons (Morgan et al., 2014). Therefore, there is a possibility that the basal TLR4 expression differs among the 5 gastrointestinal regions, especially between the ileum and the other 4 regions. Further study is needed to determine the expression level of TLR4 to confirm this hypothesis.

One of the most important receptors involved with CD is NOD2, a PRR that recognizes bacterial cell walls. Using complementary approaches, multiple groups subsequently identified mutations within the NOD2 gene on chromosome 16 as being associated with CD, but not UC. NOD2 expression is generally restricted to monocytes (Ogura et al., 2001b) and is recognized as an important mediator of inflammatory induction. The C terminus leucine-rich repeat (LRR) domain is particularly significant in that the LRR domain mediates host response to microbial stimulation for both NODs and TLRs, a separate family of proteins also mediating host responses to innate microbial signals. Activation of NOD2 results in activation of multiple signaling pathways, including the NF- $\kappa$ B and MAPK (mitogen-activated protein kinases) pathways, and ultimately leads to a variety of immune responses. TLR signaling pathways depend on MyD88 to activate NF- $\kappa$ B and MAPK to control the inflammatory response. The four identified SNPs (A1532 G, T1573C, C1688 G, and G1880 A) in the NOD2 gene may play a role in the pathogenesis of ICP in miniature dachshunds (Igarashi et al., 2015b). There is a similar report that NOD2 gene SNPs play a role in the pathogenesis of IBD in German shepherd dogs (Kathrani et al., 2014). The level of IL-1 $\beta$  expression after stimulation with a NOD2 ligand was significantly greater in monocytes from miniature dachshunds with ICP

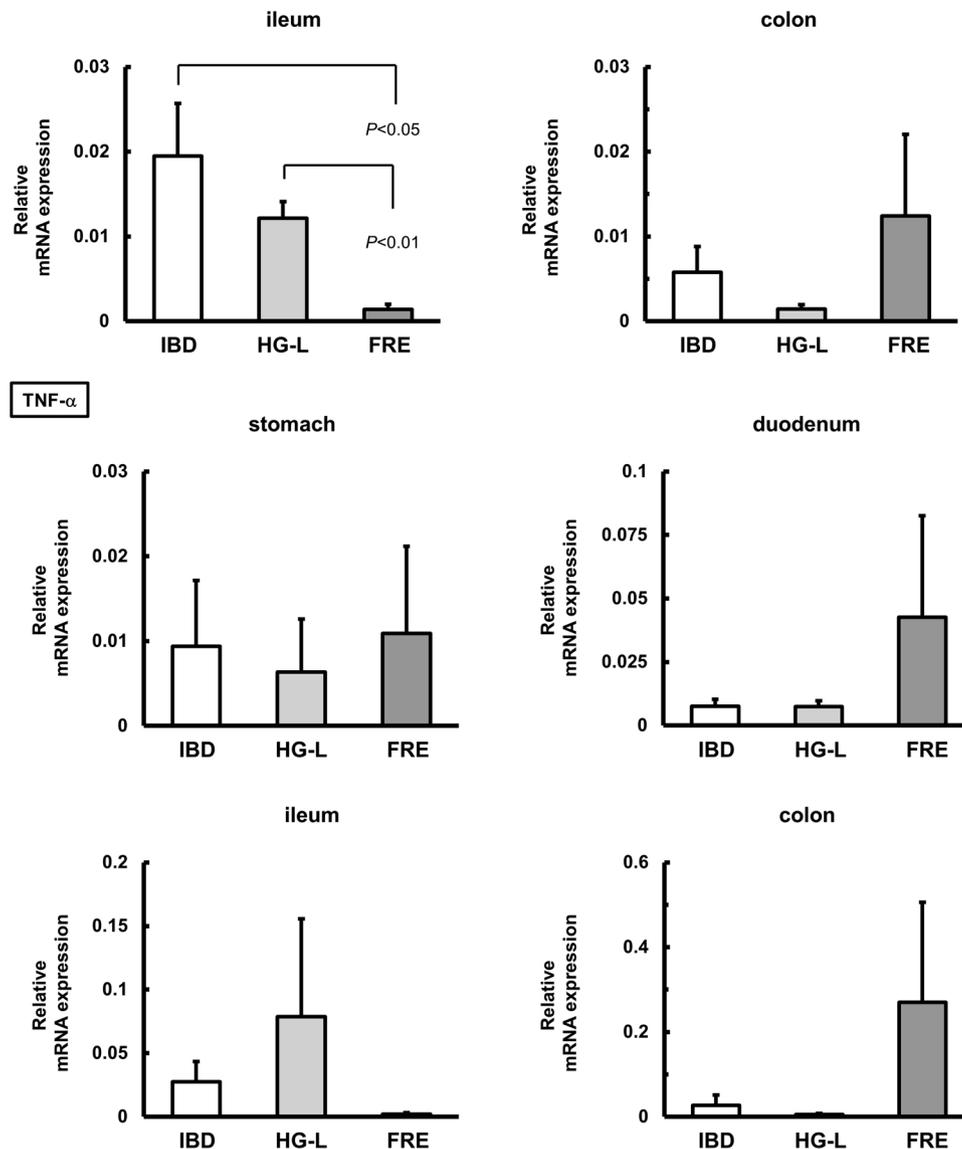


Fig. 2. (continued)

than in those from control miniature dachshunds (Igarashi et al., 2015a). NOD2 mRNA expression was greater in the descending colon of dogs with lymphocytic plasmacytic colitis than in healthy control dogs (Okanishi et al., 2013a). Similar to TLR4, levels of NOD2 were significantly upregulated in the ICP in the colon of miniature dachshunds relative to those in the controls (Igarashi et al., 2014). Also similar to TLR4, TLR ligands including LPS upregulated the expression of NOD2 in primary colonic epithelial cells (Swerdlow et al., 2006). In contrast, NOD2 mRNA expression was similar in the duodenum of dogs with IBD and control cases (Okanishi et al., 2013b). These data suggest that NOD2 as well as TLR4 may be involved in dogs with chronic gastrointestinal diseases, similar to human IBD. Based on several previous and current studies, we offer the following explanation for the relationship. TLR4 and NOD2 expression gradually increased from basal level in response to the disease severity because TLR4 and NOD2 have important roles as sensors. Interestingly, there was a positive correlation for NOD2 in only the duodenum, stomach, and rectum unlike the positive correlation for TLR4 only in the ileum. There is a possibility that TLR4 expression is different among the 5 gastrointestinal regions. Additional cases are needed to determine whether the correlation depends on the definitive diagnosis and the breed, in addition to age and gender, although the detailed mechanism under the positive correlation is

currently unknown.

Surprisingly, TNF- $\alpha$  is not associated with histopathological severity, although the levels of TNF- $\alpha$  are increased in human IBD, contributing to an inflammatory response that can be damaging. It is likely that TNF- $\alpha$  may not be involved in dogs with chronic gastrointestinal diseases, unlike human IBD. TNF- $\alpha$  has been reported to be significantly increased in diseased colonic tissue compared to non-diseased colonic tissue and colonic tissue from normal dogs (Igarashi et al., 2014). We especially focused on TNF- $\alpha$  levels in one miniature dachshund with ICP. In the stomach, duodenum, ileum, and colon, TNF- $\alpha$  levels in one miniature dachshund with ICP were low-ranked values. In contrast, TNF- $\alpha$  levels in one miniature dachshund with ICP were third highest ranked value in the rectum. Further study is needed to compare the expression levels of TNF- $\alpha$  in the healthy dogs.

Clinically, definitive diagnosis is difficult between IBD and FRE in dogs. Our findings indicated that there is a decreased level of NOD2 expression in the ileum of dogs with FRE compared to those with IBD. These findings suggest that NOD2 plays different roles in IBD and FRE.

A deficiency in our results is the lack of any controls, as have been used in the majority of previous studies evaluating TLR4 and NOD2 expression in mucosal biopsies of dogs with various forms of chronic gastrointestinal disease. In these studies, controls have included non-

diseased and diseased tissue samples from patients and/or tissue from healthy dogs, mainly beagles (Igarashi et al., 2014; Yokoyama et al., 2017a,b; Allenspach et al., 2010; McMahon et al., 2010; Burgener et al., 2008). Although TLR4 levels in normal dogs have been reported for all of the tissues examined in this study, except for the rectum (Burgener et al., 2008), NOD2 levels in normal dogs have only been reported for the colon (Igarashi et al., 2014). Further studies are needed that compare affected dogs with control dogs. A study that examines NOD2 levels will be especially informative.

The strength of our study is that we handled chronic gastrointestinal disease, including IBD and lymphoma, as one large group. In previous studies, most researchers sliced and diced chronic gastrointestinal disease. We agree that these previous approaches contributed diagnostic development. When the researchers find results in the end-definitive diagnosis, the researchers may simultaneously lose important insight into the common basis under the large group of chronic gastrointestinal disease. This is our concern regarding previous approaches. In this study, we used two characterized approaches as large (Fig. 1) and small angles (Fig. 2). The approach addressed in Fig. 1 found the correlation between the expression level of TLR4, NOD2, and TNF- $\alpha$  and the histopathological severity. Previous approaches based on the end-definitive diagnosis may not demonstrate the correlations obtained in this study. We emphasize that both approaches using the end-definitive diagnosis and the large group including the end-definitive diagnosis make sense for future study.

A limitation of our results is that dogs with chronic gastrointestinal disease in each case were not the same in terms of age (years), gender, and CCECAI. Aging dogs will spontaneously progress towards terrible and prolonged enterocolitis with diarrhea and body weight loss. Further studies will be needed to assess the correlation between TLR4 and NOD2 expression levels and age and gender of dogs. Similar to humans with IBD, genetic factors may contribute to the pathogenesis of dogs with chronic gastrointestinal disease, and an enhanced risk for the development of IBD is related to particular breeds, including Basenjis, French bulldogs, German shepherd dogs and soft-coated Wheaten terriers (German et al., 2000; Jergens et al., 1992). Further studies are needed to determine the level of TLR4 and NOD2 expression in these breeds.

Our results indicate that the increased expression of TLR4 and NOD2 is a sensor of immune responses in dogs with chronic gastrointestinal disease, and constitutes one of the several potential immune mechanisms that can contribute to histopathological severity. In conclusion, our results provide strong insight into the important roles of TLR4 and NOD2 in dogs with chronic gastrointestinal disease. Our results are also able to contribute to the relationship among IBD, HG-L, FRE, and clinical outcomes.

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