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Expression profiles of *TWEAK* and *Fn14* genes in the duodenal and colonic mucosae of dogs with chronic enteropathy

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Abstract

Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) is a cytokine that binds to fibroblast growth factor-inducible 14 (Fn14). The TWEAK/Fn14 pathway promotes intestinal inflammation, induces C-X-C motif chemokine ligand 10 (CXCL10), and is upregulated in the intestine of humans with inflammatory bowel disease. However, the role of this pathway in canine chronic enteropathy (CE) remains unclear. Therefore, we measured *TWEAK*, *Fn14*, and *CXCL10* mRNA levels in the intestinal mucosa of dogs with CE. Real-time PCR analysis revealed significantly lower *TWEAK* and *CXCL10* mRNA levels in the duodenal mucosa of dogs with antibiotic-responsive enteropathy (ARE), a type of CE, compared to healthy dogs. The findings suggested the involvement of decreased *TWEAK* and *CXCL10* mRNA levels in the ARE etiology.

Key Words: Chronic enteropathy, Dog, Tumor necrosis factor-like weak inducer of apoptosis

Dogs with chronic enteropathy (CE) present with persistent or recurrent gastrointestinal (GI) signs, such as vomiting and diarrhea, lasting for more than 3 weeks[10)]. Conventionally, CE can be classified as food-responsive enteropathy (FRE), antibiotic-responsive enteropathy (ARE), and inflammatory bowel disease (IBD) or immunosuppressant-responsive enteropathy (IRE), depending on the treatment responses[1,2,9,10,22)]. The clinical sings in dogs with FRE and ARE can be controlled by appropriate diets and antibiotics, respectively. In contrast, the management of dogs with IRE requires the long-term administration of immunosuppressive drugs. Furthermore, some dogs with CE do not respond to dietary, antibiotic, and immunosuppressive treatments, a condition called non-responsive enteropathy (NRE)[9,10)]. The pathogenesis of canine CE is complicated: aberrant intestinal immune activation against food or other antigens,

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intestinal barrier dysfunction, dysbiosis, and genetic factors are involved in chronic intestinal inflammation in dogs with CE[14,19,24]. Despite the similar clinical characteristics of each CE type, the distinct treatment responses suggest that the pathogenesis of each type may differ. However, what factors shape the etiological differences among CE remain unclear.

Tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) is a member of the TNF superfamily that binds to its specific receptor, fibroblast growth factor-inducible 14 (Fn14)[25]. TWEAK is expressed in various immune cells, including lymphocytes, macrophages, dendritic cells, and natural killer cells, and non-immune cells, including endothelial cells and neurons[13,25]. TWEAK is present on the cell surface, but its soluble form can also be released by proteolytic cleavage[25]. Fn14 is normally expressed on the surface of epithelial cells, endothelial cells, fibroblasts, and other non-hematopoietic cells at low levels in healthy tissues[13,25]. Fn14 expression is upregulated by various growth factors and inflammatory cytokines[25]. The TWEAK/Fn14 axis regulates multiple physiological processes, including cell proliferation, migration, apoptosis, and angiogenesis, and plays beneficial roles in normal tissue regeneration and repair after acute injury[13,25]. TWEAK also induces C-X-C motif chemokine ligand 10 (CXCL10) in a variety of cells, including human kidney cells, dermal fibroblasts, synoviocytes, and mouse vascular smooth muscle cells[6,15,18]. CXCL10 acts not only as a chemokine for lymphocyte recruitment but also as an antibacterial substance[8]. Thus, the TWEAK/Fn14 pathway may be involved in the protection against bacterial invasion.

Aberrant activation of the TWEAK/Fn14 pathway promotes chronic intestinal inflammation by disrupting the mucosal barrier, inducing inflammatory cell recruitment and activation, and dysregulating tissue repair and fibrosis[13]. Increased expression of TWEAK and Fn14, which reflects activation of these molecules, has been detected in the intestine of mouse models of human inflammatory bowel diseases (IBD)[12,21] and in human patients with Crohn's disease, a type of IBD[11]. In addition, the clinical and histological features in mouse models of IBD were ameliorated in TWEAK-deficient, Fn14-deficient, or anti-TWEAK antibody-treated mice compared to wild-type or control mice[12,21]. These findings indicated the pathological contribution of the TWEAK/Fn14 pathway to human IBD.

Considering the potential roles of TWEAK and Fn14 in chronic intestinal inflammation, we hypothesized that dysregulated activation of the TWEAK/Fn14 pathway might contribute to the pathogenesis of canine CE. A previous study detected *TWEAK* and *Fn14* mRNA expression in normal canine tissues, including the small intestine[26]. However, no studies have examined *TWEAK* and *Fn14* mRNA expression in dogs with CE. Therefore, in this study, we assessed these expression levels in the duodenal and colonic mucosae of healthy dogs and dogs with FRE, ARE, and IRE. Furthermore, to evaluate the possible interaction between the TWEAK/Fn14 pathway and CXCL10, we measured *CXCL10* mRNA expression levels in the intestinal mucosa of healthy dogs and dogs with each CE type.

This study included 40 dogs diagnosed with CE at Tokyo University of Agriculture and Technology Animal Medical Center between 2013 and 2019. The CE diagnostic procedure was performed according to our previous report[17]. Dogs with CE showed chronic GI signs, such as vomiting, small bowel diarrhea, and/or large bowel diarrhea, lasting for more than 3 weeks, as well as histopathological duodenitis and/or colitis in the intestinal mucosa collected by endoscopic biopsy. Other possible causes of chronic GI signs, such as metabolic diseases including electrolyte disturbances; infectious diseases including bacterial, viral, and parasitic diseases; exocrine pancreatic insufficiency; hepatic diseases; renal diseases; and neoplasms including alimentary lymphoma, were ruled out based on vaccination history and physical and clinical examinations, including blood tests, thoracic and abdominal radiography and ultrasonography, fecal tests, and endoscopic analysis described below. After the confirmation of CE, FRE was first diagnosed by the complete resolution of GI signs in a dietary

Table 1. Sequences of the oligonucleotide primers used in the present study.

Gene	Primer	Sequence (5'-3')	GenBank accession number	Size (base pairs)
TWEAK	Forward	GGAAGAGGCCAAAATCAACA	FJ915118	150
	Reverse	ACCAGCAAGTCCAGCTTCAG		
Fn14	Forward	GACCTCGACAAGTGCATGG	FJ915119	165
	Reverse	CGAGAAGCCAGAAAGCAGTC		
CXCL10	Forward	ATTGAGATGATTCCTGCAAGTCC	NM_001010949.1	85
	Reverse	TCAGACATCTTTTCTCCCCACTC		
GAPDH	Forward	CATTGCCCTCAATGACCACT	NM_001003142.2	105
	Reverse	TCCTTGGAGGCCATGTAGAC		
TBP	Forward	CTATTTCTTGGTGTGCATGAGG	XM_849432.4	96
	Reverse	CCTCGGCATTCAGTCTTTTC		
SDHA	Forward	GCCTTGGATCTCTTGATGGA	XM_535807.5	92
	Reverse	TTCTTGGCTCTTATGCGATG		
HMBS	Forward	TCACCATCGGAGCCATCT	XM_546491	112
	Reverse	GTTCCCACCACGCTCTTCT		

GAPDH, *TBP*, and *SDHA* were used as reference genes for the duodenal mucosa; *GAPDH*, *TBP*, and *HMBS* were used as reference genes for the colonic mucosa.
TWEAK, tumor necrosis factor-like weak inducer of apoptosis; *Fn14*, fibroblast growth factor-inducible molecule 14; *CXCL10*, *C-X-C* motif chemokine ligand 10; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *TBP*, TATA-binding protein; *SDHA*, succinate dehydrogenase complex, subunit A; *HMBS*, hydroxymethylbilane synthase.

trial using hydrolyzed or novel protein diets. Then, ARE was diagnosed by the complete resolution of GI signs in response to antibiotic treatment with metronidazole and/or tylosin. After the exclusion of FRE and ARE, IRE was diagnosed based on the clinical improvement of GI signs with prednisolone. The clinical severity of CE in the 40 dogs was scored according to the canine chronic enteropathy clinical activity index (CCECAI)[3]. The control group comprised nine healthy intact male beagles (median age, 3.7 years; range, 0.3–6.4 years; median body weight, 11.3 kg; range, 8.1–12.6 kg). All procedures were approved by the Institutional Animal Care and Use Committee of Tokyo University of Agriculture and Technology (approval numbers: 28-34 and 30-132). Informed consent was obtained from the owners of the dogs included in this study.

The duodenum and colon were endoscopically examined as we described previously[17,20]. Using endoscopic biopsy forceps, duodenal mucosa was collected from all dogs; colonic mucosa was obtained from eight of nine healthy control dogs and 33 of 40 dogs with CE. More than six specimens were obtained from each region. The duodenal and colonic biopsy specimens of dogs with CE were histopathologically analyzed and graded by a board-certified veterinary anatomic pathologist (HK, American College of Veterinary Pathologists) according to the guideline of the World Small Animal Veterinary Association (WSAVA) international GI standardization group[24]. A portion of each biopsy sample was immediately stored in RNAlater® solution (Thermo Fisher Scientific, Waltham, MA, USA) until RNA extraction.

Real-time PCR was performed using a Thermal Cycler Dice® Real Time System *Lite* (Takara Bio, Shiga, Japan) as previously described[16,20]. The primers for real-time PCR (Table 1) were designed according to previous reports[7,26]. As the reference genes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), TATA-binding protein (TBP), and succinate dehydrogenase complex, subunit A were used for the duodenal mucosa, while GAPDH, TBP, and hydroxymethylbilane synthase were used for the colonic mucosa (Table 1) according to our previous report[23]. The relative mRNA expression levels of the target genes were determined using the $2^{-\Delta Ct}$

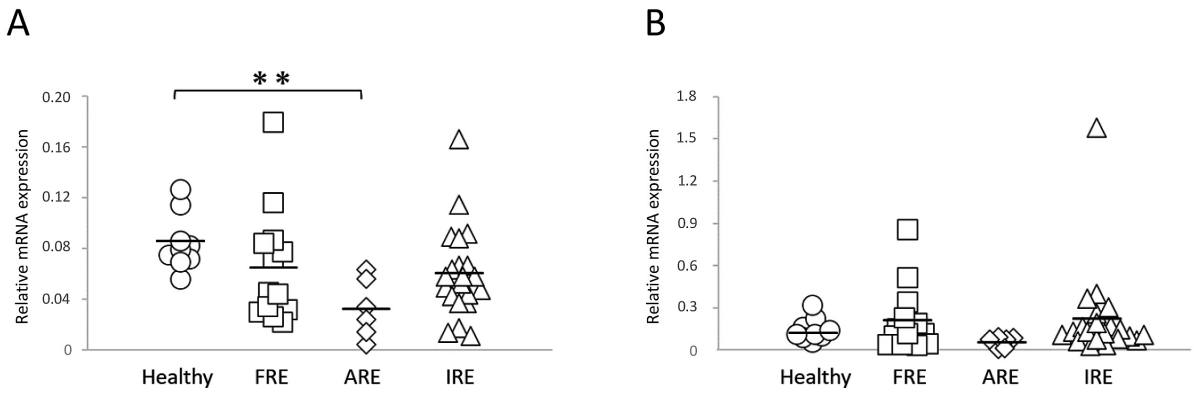

Fig. 1. Relative mRNA expression levels of *TWEAK* and *Fn14* in the duodenal mucosa of dogs with chronic enteropathy (CE). The expression levels of *TWEAK* (A) and *Fn14* (B) mRNA were analyzed by real-time PCR in healthy dogs (n = 9) and dogs with CE (n = 40), including dogs with food-responsive enteropathy (FRE) (n = 13), antibiotic-responsive enteropathy (ARE) (n = 6), and immunosuppressant-responsive enteropathy (IRE) (n = 21). The horizontal lines in each group represent the mean values. The relative mRNA expression levels were compared among healthy dogs and dogs with FRE, ARE, or IRE, using the Kruskal–Wallis test, followed by the Steel test. *P < 0.01.

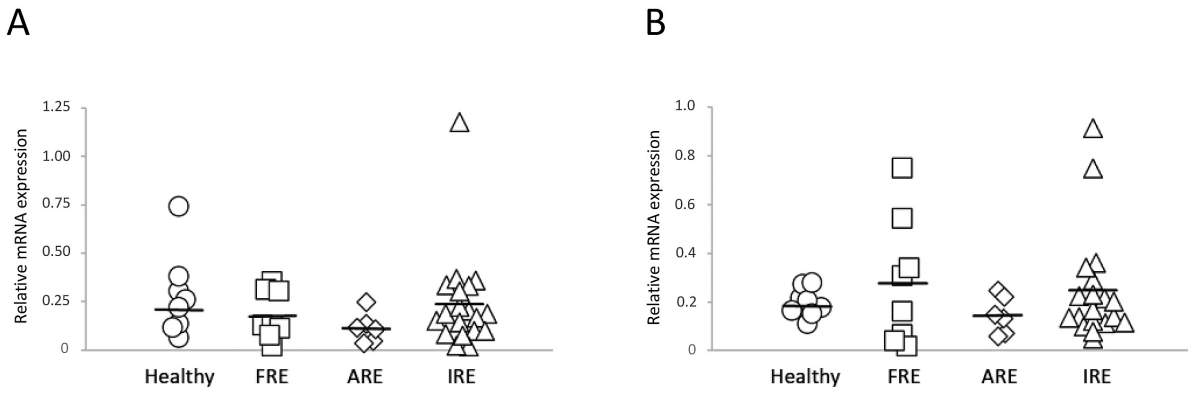

Fig. 2. Relative mRNA expression levels of *TWEAK* and *Fn14* in the colonic mucosa of dogs with chronic enteropathy (CE). The expression levels of *TWEAK* (A) and *Fn14* (B) mRNA were analyzed by real-time PCR in healthy dogs (n = 8) and dogs with CE (n = 33), including dogs with food-responsive enteropathy (FRE) (n = 8), antibiotic-responsive enteropathy (ARE) (n = 6), and immunosuppressant-responsive enteropathy (IRE) (n = 19). The horizontal lines in each group represent the mean values. The relative mRNA expression levels were compared among healthy dogs and dogs with FRE, ARE, or IRE, using the Kruskal–Wallis test.

method, wherein each value was presented as an n-fold difference relative to the geometric mean of the three reference genes.

The normality of all data was analyzed using the Shapiro–Wilk test. Data among four groups were compared using the Kruskal–Wallis test, followed by the Steel test. The correlations between two parameters were evaluated using the Spearman's rank correlation coefficient (r_s). Statistical analysis was performed using BellCurve for Excel software version 3.20 (Social Survey Research Information Co., Ltd., Tokyo, Japan). $P < 0.05$ was considered statistically significant.

The clinical and histopathological characteristics of the 40 dogs with CE are summarized in Supplementary Table 1. The median age of the dogs with CE was 8 years (range, 1.8–14.1 years), and the median body weight was 5.7 kg (range, 1.8–29.7 kg). The median CCECAI score at the first visit was 6 (range, 0–16). The median WSAVA scores in the duodenum and colon were 9 (range, 3–17) and 5 (range, 1–9), respectively. Among the 40 dogs with CE, 13, 6,

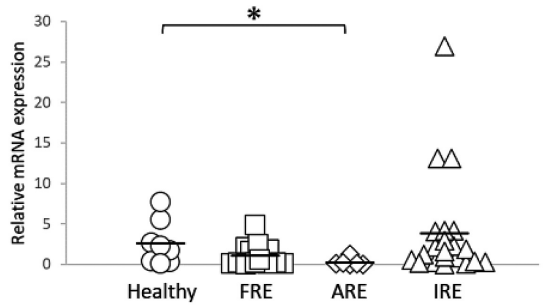

Fig. 3. Relative mRNA expression levels of *CXCL10* in the duodenal mucosa of dogs with chronic enteropathy (CE). The expression levels of *CXCL10* mRNA were analyzed by real-time PCR in healthy dogs (n = 9) and dogs with CE (n = 40), including dogs with food-responsive enteropathy (FRE) (n = 13), antibiotic-responsive enteropathy (ARE) (n = 6), and immunosuppressant-responsive enteropathy (IRE) (n = 21). The horizontal lines in each group represent the mean values. The relative mRNA expression levels were compared among healthy dogs and dogs with FRE, ARE, or IRE, using the Kruskal–Wallis test, followed by the Steel test. *P < 0.05.

and 21 were diagnosed with FRE, ARE, and IRE, respectively.

TWEAK and *Fn14* mRNA expression levels in the duodenal and colonic mucosae were compared among healthy dogs and dogs with FRE, ARE, or IRE. In the duodenal mucosa, *TWEAK* mRNA expression levels were significantly lower in dogs with ARE compared to healthy dogs (Fig. 1A; P = 0.0088), while *Fn14* mRNA expression levels did not differ significantly among healthy dogs and dogs with FRE, ARE, or IRE (Fig. 1B; P > 0.05). In the colonic mucosa, *TWEAK* and *Fn14* mRNA expression levels did not differ significantly among healthy dogs and dogs with FRE, ARE, or IRE (Fig. 2A, B; P > 0.05, respectively).

Since TWEAK induces the antibacterial chemokine CXCL10, we measured *CXCL10* mRNA expression levels in the duodenal mucosa of healthy dogs and dogs with FRE, ARE, or IRE. As shown in Fig. 3, *CXCL10* mRNA expression levels were significantly lower in dogs with ARE than in healthy dogs (P = 0.0475).

To examine whether decreased *TWEAK* and *CXCL10* mRNA expression levels in the duodenal mucosa of dogs with ARE were associated with clinical and histopathological severity, we assessed the correlations between the gene expression levels and CCECAI or WSAVA score in the duodenum of dogs with ARE. We detected no significant correlations between *TWEAK* mRNA expression levels and CCECAI (r_s = –0.5657, P = 0.2420) or WSAVA score (r_s = 0.4630, P = 0.3551) and between *CXCL10* mRNA expression levels and CCECAI (r_s = 0.3769, P = 0.4615) or WSAVA score (r_s = –0.1471, P = 0.7809) in the duodenal mucosa of dogs with ARE. We further analyzed the relationship between *TWEAK* and *CXCL10* mRNA expression levels in the duodenum of dogs with ARE. However, we observed no significant correlations between *TWEAK* and *CXCL10* mRNA expression levels in the duodenal mucosa of dogs with ARE (r_s = –0.7714, P = 0.0724).

In this study, dogs with various ages were enrolled. Thus, we analyzed effects of age on *TWEAK* and *CXCL10* mRNA expression levels in the duodenal mucosa of healthy dogs and dogs with CE. However, we found no significant correlations between age and *TWEAK* or *CXCL10* mRNA expression levels in the duodenal mucosa of the dogs (Supplementary Table 2).

The present study demonstrated significantly lower *TWEAK* mRNA expression levels in the duodenal mucosa of dogs with ARE compared to healthy dogs. In contrast, *Fn14* mRNA expression levels in the duodenal mucosa and *TWEAK* and *Fn14* mRNA expression levels in the colonic mucosa did not differ significantly among healthy dogs and dogs with FRE, ARE, or IRE. Furthermore, *CXCL10* mRNA expression levels were significantly lower in the duodenal mucosa of dogs with ARE compared to healthy dogs. These findings suggest that decreased *TWEAK* and *CXCL10* mRNA expression levels might be involved in the pathogenesis of duodenitis in dogs with ARE.

The GI signs in dogs with ARE can be managed with antibiotic treatment, suggesting a pivotal role of intestinal dysbiosis in the pathogenesis of ARE. A recent study reported the significant difference in the beta, but not the alpha-diversity, of intestinal microbiota between healthy dogs and dogs with ARE[4]. In addition, tylosin significantly changed the alpha-diversity with a reduction in the abundance of

intestinal microbiota in dogs with ARE[4]. Although no specific characteristics shaping intestinal dysbiosis have been identified in dogs with ARE[4], the findings of this study suggest that an impaired TWEAK/Fn14 pathway might be associated with intestinal dysbiosis through reduced production of the antibacterial chemokine CXCL10 in dogs with ARE. However, the present study did not demonstrate the correlations between *TWEAK* and *CXCL10* mRNA expression levels in the duodenal mucosa of dogs with ARE. Furthermore, it is unclear whether TWEAK could induce CXCL10 production in the intestinal mucosa of dogs. Thus, our hypothesis regarding the role of decreased TWEAK and CXCL10 levels in the etiology of ARE should be addressed in further studies.

The indiscriminate use of antibiotics increases the risk of antibiotic resistance in both individual patients and public health. Thus, it was recently proposed that the diagnostic use of antibiotics for the CE classification should be avoided, and if necessary, should be performed only after histopathologic evaluation of intestinal mucosa and after the exclusion of FRE and IRE based on appropriate therapeutic trials using diets and immunosuppressive drugs in dogs with CE[5]. Because the dogs with CE in this study were collected prior to the publication of the proposal, the diagnostic flow of the CE classification in this study differed from that in the proposal. In the present study, antibiotic trials were performed after histopathologic evaluation of GI biopsies and after unsuccessful dietary trials that excluded FRE but before the administration of immunosuppressive drugs for the diagnosis of IRE. Thus, we cannot exclude the possibility that some of the ARE dogs in this study might have responded to immunosuppressive drugs and were classified as IRE according to the recently proposed diagnostic algorithm[5].

The etiologies of canine CE and human IBD are similar, in which dysregulated intestinal immune activation, dysbiosis, intestinal barrier dysfunction, and genetic factors contribute to the development of these two diseases. However, as canine CE may not be a counterpart to human IBD[9], the similarities and differences between the two diseases should be clarified. Previous studies have indicated abnormal activation of the TWEAK/Fn14 pathway in the GI tract of human IBD[11] and mouse models of IBD[12,21], as revealed by the upregulated expression of these molecules. In contrast, the results of the present study did not demonstrate increased *TWEAK* and *Fn14* mRNA expression levels in the duodenal and colonic mucosae of dogs with each CE type. These findings suggest that the TWEAK/Fn14 pathway plays different roles in the pathogenesis of canine CE and human IBD.

In the current study, the expression profiles of *TWEAK* and *CXCL10* mRNA in the duodenal and colonic mucosae differed among dogs with CE, suggesting that the TWEAK/Fn14 pathway and CXCL10 may play different roles in the pathogenesis of FRE, ARE, and IRE. These expression profiles suggest that decreased TWEAK and CXCL10 levels may contribute to duodenitis in dogs with ARE. In contrast, these molecules may not be involved in the development of duodenitis in dogs with FRE and IRE and colitis in dogs with FRE, ARE, and IRE.

This study has several limitations. First, *TWEAK*, *Fn14*, and *CXCL10* were measured at the mRNA level. To clarify the distribution and cellular sources of the TWEAK/Fn14 pathway and CXCL10, the protein expression of these molecules should be examined by immunohistochemical analysis using appropriate antibodies. Second, the number of dogs with CE, especially those with ARE, was relatively small. In addition, histopathological differences in the intestine might have affected the gene expression levels in each CE type. These should be analyzed using a larger population of dogs with CE. Third, dogs with NRE could not be included in this study due to the lack of sample availability. Fourth, the control group included only healthy, intact male beagles. As an appropriate control group, age-, sex-, and breed-matched healthy dogs should be used. These limitations should be addressed in future studies.

In conclusion, the present study demonstrated decreased *TWEAK* and *CXCL10* mRNA expression levels in the duodenal mucosa of dogs with ARE. The findings suggest that TWEAK-mediated

reduction in the antibacterial chemokine CXCL10 might be associated with intestinal dysbiosis in the duodenal mucosa of dogs with ARE. Further studies are warranted to elucidate how decreased *TWEAK* and *CXCL10* mRNA expression levels contribute to the development of duodenitis in dogs with ARE.

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Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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