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Gene expression of leucine-rich alpha-2 glycoprotein in the polypoid lesion of inflammatory colorectal polyps in miniature dachshunds

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ABSTRACT. Inflammatory colorectal polyps (ICRPs) in miniature dachshunds (MDs) are speculated to be a breed-specific inflammatory bowel disease (IBD). Leucine-rich alpha-2 glycoprotein (LRG) has been identified as a novel biomarker of human IBD. The aim of this study was to examine *LRG* gene expression in the polypoid lesions of ICRPs. Polypoid lesion specimens were collected from 24 MDs with ICRPs. Nonpolypoid colonic mucosa was collected from 18 MDs with ICRPs and 10 controls. The gene expression of *LRG*, *interleukin* (*IL*)*-1β*, *IL-6*, *tumor necrosis factor-α*, and *IL-22* was examined. The expression of *LRG* gene was significantly increased in the polypoid lesions of ICRPs and correlated with that of the four cytokines. In conclusion, the *LRG* gene was expressed within the polypoid lesions of ICRPs and might be associated with local cytokine expression.

KEY WORDS: inflammatory colorectal polyp, inflammatory cytokine, leucine-rich alpha-2 glycoprotein, miniature dachshund

Inflammatory colorectal polyps (ICRPs) are one of the common causes of large bowel diarrhea in miniature dachshunds (MDs) in Japan [10]. ICRPs in MDs cause the development of multiple small polyps and/or large solitary polyp in the colorectal area and are histopathologically characterized by increased numbers of goblet cells with dilated crypts, and infiltration of inflammatory cells, predominantly neutrophils and macrophages [10, 19]. MDs with ICRPs demonstrate a good response to immunosuppressive therapies, such as prednisolone, cyclosporine and leflunomide [3, 10]. Therefore, ICRPs in MDs are speculated to be a novel, breed-specific form of canine inflammatory bowel disease (IBD) [6, 11].

Leucine-rich alpha-2 glycoprotein (LRG), which was first isolated from human serum in 1977, is a glycoprotein of approximately 50 kDa that contains repetitive sequences and a leucine-rich motif [17]. LRG has been reported to be expressed by the hepatocytes and neutrophils as well as epithelial cells, such as lung epithelial cells and intestinal epithelial cells [5, 9, 13, 16]. Although the function of LRG has remained unclear for many decades, it is known that the LRG protein is secreted during the acute phase of inflammation in response to inflammatory cytokines, such as interleukin (IL)-6, IL-1β, tumor necrosis factor (TNF)-α, and IL-22 [13, 16]. Recently, serum LRG has been identified as a novel biomarker for evaluating disease activity in several inflammatory disorders in human, such as rheumatoid arthritis, systemic lupus erythematosus, juvenile idiopathic arthritis and psoriasis [1, 4, 8, 12, 14]. The serum LRG concentrations were significantly elevated in patients with rheumatoid arthritis and found to be correlated with disease activity [4].

LRG has also been reported to be a novel inflammatory biomarker of human inflammatory bowel diseases, such as Crohn's disease (CD) and ulcerative colitis (UC) [12, 13]. The serum LRG concentrations were significantly elevated in patients with active CD and UC, and the serum LRG concentrations were more closely correlated with disease activity in UC than the serum C-reactive protein (CRP) concentrations [12, 13]. In addition, the regulation of LRG production appears to differ from that of CRP because LRG production can be induced in an IL-6-independent manner [13]. Furthermore, the expression of LRG was increased

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in the inflamed colonic tissues of UC patients, indicating the local production of LRG in the inflamed colonic mucosa of patients with UC [13].

These findings suggested that LRG production might increase in the polypoid lesions of MDs with ICRPs, similar to its production in humans with UC. Thus, the aim of this study was to examine *LRG* mRNA expression in the polypoid lesions of MDs with ICRPs and to examine the correlation of *LRG* mRNA expression with inflammatory cytokine mRNA expression.

Colorectal mucosal specimens were obtained from 24 MDs at Hokkaido University Veterinary Teaching Hospital between April 2010 and August 2017 to investigate the hematochezia, tenesmus and increased defecation frequency of the these MDs. Colorectal mucosal specimens were obtained from 22 MDs with ICRPs by colonoscopy and 2 MDs with ICRPs by mucosal pull-through surgical excision. All the dogs were diagnosed with ICRPs based on the clinical and histopathological findings described in a previous study [10, 19]. Seven MDs with ICRPs had received prednisolone treatment (0.4 mg/kg every other day to 1.8 mg/kg/day) until the day of the endoscopic examination and biopsy. In addition, cyclosporine had also been used in two MDs with ICRPs. The median age of these dogs was 9.5 years (range, 6–14 years), and the MDs included 15 males (10 intact and 5 neutered) and 9 females (1 intact and 8 neutered). The median body weight was 5.3 kg (range, 3.5–7.7 kg). No distinct abnormalities were detected in these cases by a hematological, serum biochemical, and fecal examination or by abdominal ultrasound, except for increased serum CRP concentrations (>1 mg/d*l*) in 12 dogs. Informed consent was obtained from the owners of the dogs enrolled in this study.

In addition, colonic mucosal samples were obtained endoscopically from 10 control dogs consisting of 4 laboratory beagles and 6 mongrels. There were 3 intact males and 7 intact females, and the median ages of these dogs was 2.5 years (range, 1–9 years). No dog had any clinical signs or evidence of weight loss for more than one year before the endoscopic procedure. The hematological, serum biochemical, fecal, and abdominal ultrasound examinations revealed no abnormalities in any dogs. The use of the dogs in this study was approved by the Animal Care and Use Committee, Graduate School of Veterinary Medicine, Hokkaido University (approval no. 13-0142).

A colonoscopy was performed in 22 dogs with ICRPs and 10 control dogs under general anesthesia using a VQ-8143B flexible video endoscope (AVS, Tokyo, Japan). Multiple mucosal biopsies (8–10) weighing 5–10 mg each were collected for diagnosis and gene expression analysis using VQ-143Q-B53 biopsy forceps (AVS) from the polypoid lesions of 22 dogs with ICRPs. Multiple mucosal biopsies were also collected from the macroscopically normal descending colon (nonpolypoid lesions), which was located 10–15 cm from the polypoid lesion, from 18 dogs with ICRPs. In the control dogs, multiple mucosal biopsies were obtained from the descending colon. For histological analysis, biopsy samples were fixed in 10% neutral buffered formalin, embedded in paraffin wax, and routinely processed for hematoxylin-eosin staining. These biopsy specimens were assessed by a board-certified pathologist (YK) according to the World Small Animal Veterinary Association (WSAVA) guidelines [2]. One of the colorectal mucosal specimens was placed in RNA*later* solution (Ambion Inc., Austin, TX, USA), incubated at 4°C for 24 hr and then transferred to −80°C.

The total RNA was extracted from the polypoid and nonpolypoid lesion specimens using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions and the genomic DNA was removed from the samples with a commercially available kit (RNase-Free DNase set; Qiagen). cDNA was synthesized from 0.5 μg of total RNA using ReverTra Ace (Toyobo, Osaka, Japan).

Quantitative PCR (qPCR) was performed to assess *LRG*, *IL-1β*, *IL-6*, *IL-22* and *TNF-α* gene expression. For accurate quantification, hydroxymethyl-bilane synthase *(HMBS*), ribosomal protein L32 (*RPL32*) and ribosomal protein S18 (*RPS18*) were chosen as the reference genes as previously described [6, 7]. The sequences of the primer pair for *LRG* were as follows: 5′-ACGACCTCTATCGGTGGCTTG-3′ (forward) and 5′-TCAGTGTGACTCGGCTGCTTC-3′ (reverse). The sequences of the primer pairs used for the cytokines, except for *IL-22*, for qPCR were obtained from previous reports [6, 21]. The sequences of the primer pair for *IL-22* were as follows: 5′- AGTCCAACTTCCAGCAGCCCTA-3′ (forward) and 5′-CCCATATTGACTCCGTGGAACAG -3′ (reverse). Each qPCR reaction was performed in 20 μl and contained 500 nM each primer, 1 μl cDNA, and FastStart Essential DNA Green Master (Roche Diagnostics, Mannheim, Germany). qPCR was conducted using a LightCycler Nano (Roche Diagnostics). The amplification conditions were 95°C for 10 min and 45 cycles of PCR (95°C for 10 sec, 60°C for 10 sec and 72°C for 15 sec), followed by dissociation (95°C for 10 sec, 65°C for 60 sec and 95°C for 1 sec). All the samples were examined in duplicate, and each PCR included a nontemplate control. The reactions efficiency was determined for each primer set using 10-fold dilutions (10^8 molecules μl^{-1} to 10^2 molecules μl^{-1}) of plasmids ligated with the *LRG*, cytokine and reference genes, and the correlation coefficient was >0.98. The cycle threshold values that indicated the point at which the amplification curves of the PCR intersected with the threshold were determined using LightCycler Nano Software 1.0 (Roche Diagnostics). The melting curve analysis did not show misprinting in any of the reactions.

Gene expression was quantified by averaging the absolute gene copy number for each duplicate sample, followed by the normalization of the expression of each target gene to the geometric mean of the three reference genes.

The statistical analyses were performed using JMP 13 (SAS Institute Inc., Cary, NC, USA). The normality distribution was assessed by the Shapiro-Wilk *W* test. The Kruskal-Wallis test was used to test for overall differences in *LRG* and cytokine mRNA expression among the polypoid lesions of ICRPs, the nonpolypoid lesions of ICRPs and the colonic mucosa of control dogs. The Steel-Dwass test was used to test for between-group differences. The relationships between *LRG* mRNA expression and cytokine mRNA expression were evaluated using Spearman's rank correlation coefficient. A value of $P<0.05$ was considered significant.

Twenty-four MDs were diagnosed with ICRPs by the histopathological analysis of polypoid lesion specimens according to the previous reports [10, 19]. Two MDs had diffused multiple small polyps in the colorectal region, 6 MDs had large solitary polyp, and the remaining 16 MDs had both large and small polyps. Biopsy specimens of the polypoid lesions were obtained from multiple small polyps

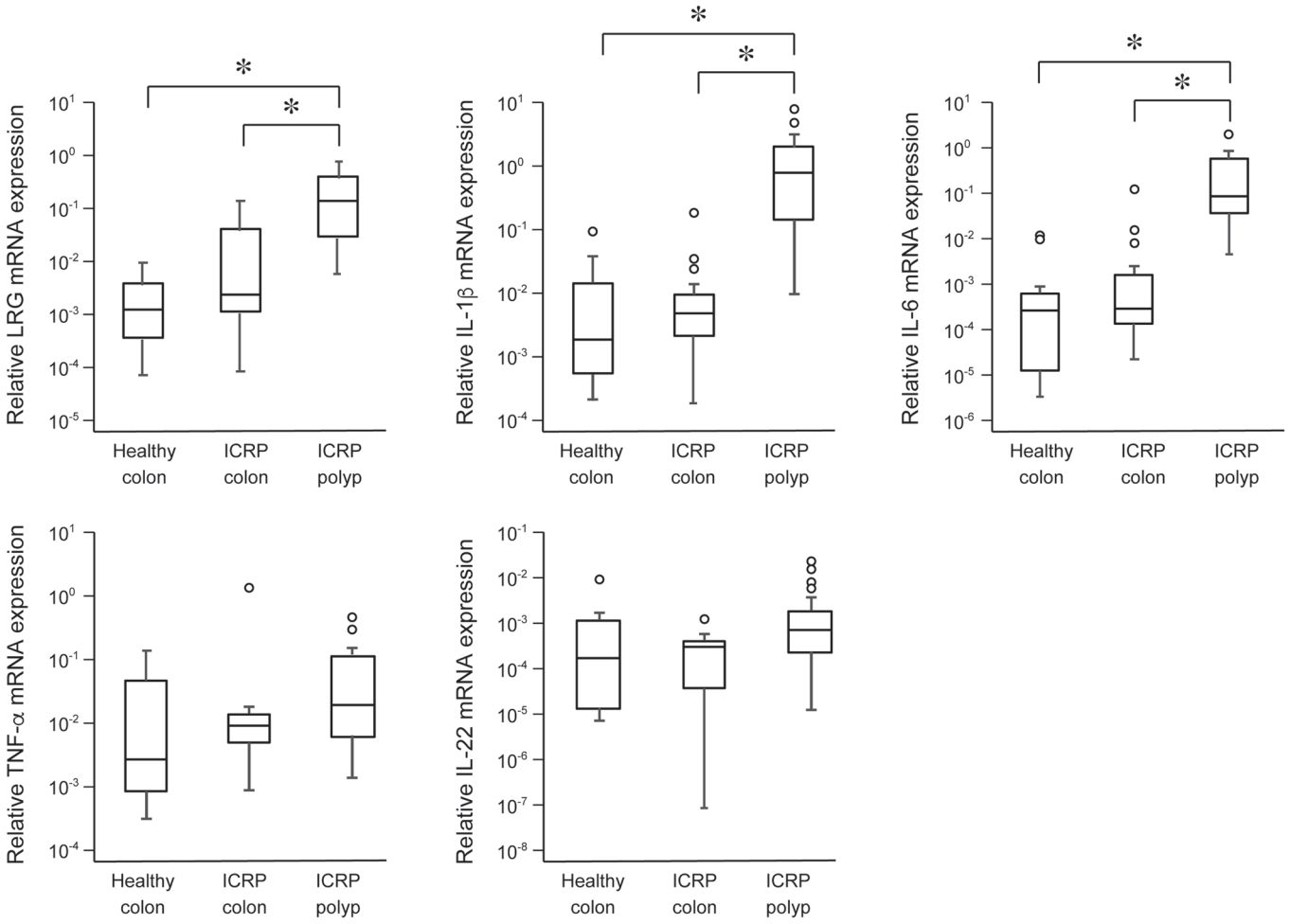

Fig. 1. The relative expression levels of *leucine-rich alpha-2 glycoprotein* (*LRG*), *interleukin* (*IL*)*-1β*, *IL-6*, *tumor necrosis factor* (*TNF*)*-α*, and *IL-22* mRNA in the colonic mucosa of healthy controls (Healthy colon, n=10) and nonpolypoid lesions (ICRP colon, n=18) and polypoid lesions (ICRP polyp, n=24) of ICRPs. The transcription levels of *LRG*, *IL-1β*, *IL-6*, *TNF-α* and *IL-22* were normalized to the transcription levels of *HMBS*, *RPL32* and *RPS18*. The top and bottom of the box represent the 75th and 25th percentiles, respectively; the middle line represents the median; the whiskers indicate the highest and lowest data points within 1.5 times the length of the quartiles; and the circles represent outliers. The asterisks indicate significant differences (*P<0.01).

in 2 dogs and large polyp in 22 dogs. The nonpolypoid lesion specimens from 18 MDs with ICRPs and the colonic mucosal specimens from 10 control dogs were determined to be histologically normal according to the WSAVA guidelines [2]. The relative expression of *LRG* mRNA in the polypoid lesions of ICRPs was significantly increased compared to that in the nonpolypoid lesions of ICRPs and the colonic mucosa of control dogs (P<0.01) (Fig. 1). In addition, the relative expression of *IL-1β* and *IL-6* mRNA in the polypoid lesions of ICRPs was significantly increased compared to that in the nonpolypoid lesions of ICRPs and the colonic mucosa of control dogs (P<0.01) (Fig. 1). Although, the *TNF-α* and *IL-22* gene expression levels among the three groups showed different tendencies, these differences were not statistically significant (P=0.057 and P=0.053, respectively). As shown in Table 1, the mRNA expression of *LRG* was positively correlated with the mRNA expression of all four cytokines mRNA expressions. The most relevant association was found between the mRNA expression of *LRG* and *IL-1β* (r_s=0.8539, P<0.001).

Table 1. Correlation between *leucine-rich alpha-2 glycoprotein* and *cytokines* mRNA expression levels

	Leucine-rich alpha-2 glycoprotein	
	r_s	P-value
IL-1β	0.8922	<0.001
IL-6	0.8243	<0.001
TNF-α	0.8191	<0.001
IL-22	0.6678	0.004

IL: interleukin, TNF: tumor necrosis factor.

In human UC, the serum LRG concentrations are elevated in patients with active UC compared with patients in remission and healthy controls and are more closely correlated with disease activity than the serum CRP concentrations [13]. In addition, the serum LRG levels were significantly correlated with endoscopic activities and are expected to be a novel biomarker for detecting mucosal healing in patients with UC [15]. Serum LRG is thought to be derived from not only hepatocytes, but also neutrophils

and epithelial cells within the inflammatory lesions [9, 13, 16]. Local production of the LRG protein, in addition to that from hepatocytes, is a unique feature of LRG compared to other acute phase proteins, such as CRP. In dogs with ICRPs, *LRG* gene expression was significantly increased in the polypoid lesion, indicating the local production of the *LRG* gene in the polypoid lesions of ICRPs similar to its production in human UC.

CRP is one of the most widely used acute phase proteins whose synthesis in hepatocytes is stimulated by IL-6. In contrast, the induction of LRG in human hepatoma HepG2 cells was stimulated by not only IL-6, but also IL-1β and TNF-α [16]. This previous study also reported that the induction of LRG by IL-6 was synergistically upregulated by either IL-1β or TNF-α. In addition, the synthesis of LRG in colonic adenocarcinoma COLO205 cells was stimulated by IL-6, TNF-α and IL-22 in a dose-dependent manner [13]. Furthermore, the serum LRG levels were increased in IL-6-deficient mice with LPS-mediated acute inflammation [13]. The results of these previous studies indicated that the synthesis of LRG is induced by not only IL-6 but also various inflammatory cytokines, such as IL-1β, TNF-α and IL-22. In fact, *LRG* mRNA expression was well correlated with not only *IL-6* mRNA expression but also *IL-1β*, *TNF-α* and *IL-22* mRNA expression in the polypoid lesions of MDs with ICRPs, although *TNF-α* and *IL-22* mRNA expression was not significantly increased in the polypoid lesions of MDs with ICRPs. The results of the current study indicated that *LRG* mRNA production in the polypoid lesions was possibly stimulated by four cytokines within the polypoid lesions of MDs with ICRPs.

Although the current study did not examine the source of *LRG* mRNA expression, neutrophils and colonic epithelial cells within the polypoid lesions might be sources of *LRG* gene expression. Previous studies indicated that in addition to hepatocytes, LRG could be produced by neutrophils [9] and intestinal epithelial cells [13]. The identification of LRG-producing cells localized within the polypoid lesions of MDs with ICRPs by immunohistochemistry or *in situ* hybridization is needed in future studies.

Although the function of LRG remains unclear, a previous study reported that LRG promotes T helper 17 (Th17) cell differentiation and arthritis development in a mouse model of collagen-induced arthritis [20]. In previous studies, the gene expression of *IL-17* was found to be upregulated in the polypoid lesions of MDs with ICRPs [7, 11]. IL-17, which is a major pro-inflammatory cytokine produced by Th17 cells, has been shown to induce neutrophil infiltration and has been reported to play an important role in severe neutrophil accumulation within polypoid lesions, which are histopathological characteristics of MDs with ICRPs [10, 11]. Thus, it is possible that *LRG* gene upregulation within polypoid lesions promotes Th17 cell differentiation and IL-17 production, which results in the exacerbation of the polypoid lesions of MDs with ICRPs.

In the previous studies, *IL-1β*, *IL-6*, *TNF-α* and *IL-22* gene expression was significantly increased in the polypoid lesions of MDs with ICRPs compared with that in the control colonic mucosa of healthy dogs [6, 7, 18]. The current study also demonstrated upregulated *IL-1β* and *IL-6* gene expression within the polypoid lesions of dogs with ICRPs. In contrast, *TNF-α* and *IL-22* gene expression was not different among groups. Although the reason for the difference in *TNF-α* and *IL-22* gene expression between the current study and the previous studies was unknown, the prior administration of prednisolone might affect *TNF-α* and *IL-22* gene expression within the polypoid lesions. Seven dogs out of the 24 dogs with ICRPs were treated with prednisolone for several days prior to visiting our teaching hospital. However, *TNF-α* and *IL-22* gene expression was not different among groups even when we examined these gene expression using 17 ICRP dogs without prior administration of prednisolone (data not shown). Another explanation is that the number of dogs with ICRPs and control dogs used for qPCR analysis and the composition of the polypoid lesions (solitary large polyp or multiple small polyps) were different from those used in previous studies [6, 7, 18].

There are several limitations in our study. First, we examined only gene expression, but not protein expression, within the polypoid lesions of MDs with ICRPs. Thus, it is necessary to examine protein expression using canine LRG-specific antibodies or antibodies that cross-react with canine LRG. Second, we did not examine the serum LRG levels in the MDs with ICRPs. In future studies, it is necessary to establish a canine-specific immunoassay, such as an enzyme-linked immunosorbent assay, to measure the serum LRG levels in dogs with ICRPs. Third, we only examined the polypoid lesions from dogs with ICRPs and did not examine the intestinal mucosa from dogs with other inflammatory or neoplastic diseases. Thus, it is not known whether increased *LRG* mRNA expression in the colorectal mucosa is specific to ICRPs. Fourth, since there are no established criteria for the accurate quantification of the histopathological severity of the polypoid lesions of ICRPs using endoscopic biopsy samples, we could not examine the correlation between LRG gene expression and histopathological severity. Thus, in future studies, it is necessary to examine the correlation between LRG protein or mRNA expression by immunohistochemistry or *in situ* hybridization and histopathological severity using surgically excised polypoid lesions of ICRPs. Finally, since the control dogs mainly consisted of female, young adult dogs, the gender and age of the control dogs were not matched to those of the MDs with ICRP.

In conclusion, we have shown that *LRG* mRNA expression was increased in the polypoid lesions of MDs with ICRPs and was well correlated with inflammatory cytokine mRNA expression. The local production of the *LRG* gene indicated the possibility that the LRG protein may be a novel biomarker of intestinal inflammation in dogs with intestinal disorders.

CONFLICT OF INTEREST. The authors declare no conflicts of interest.

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