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Atopic dog skin shows decrease of claudin-1 but increase of atopic signature cytokines

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Abstract: Canine atopic dermatitis is a common allergic skin disease in dogs caused by defects in immunological and epidermal barriers. Claudin-1, a protein present in tight junctions, is associated with many skin conditions. Our aim was to investigate expression levels of claudin-1 and atopic signature cytokines in atopic and normal dog skins. The affected skin showed significantly decreased intensity and epidermal distribution of claudin-1 compared to the normal control skin ($P < 0.05$). The CD3+/CD4+ and CD3+/CD8+ T cell subsets, eosinophils, and neutrophils infiltrated the affected epidermis and dermis. The CD3+/CD4+ T cell subsets in lesional skin were significantly higher than CD3+/CD8+ T cell subsets. *CLDN-1* mRNA expression was markedly downregulated in the lesional skin, while *IL-17A* was upregulated in both lesional and nonlesional skin. *IL-4* and *IL-31* mRNA was upregulated in the lesional skin, and *TNF-α* mRNA was significantly upregulated in the nonlesional skin ($P < 0.01$ and $P < 0.05$, respectively). Correlation-based hierarchical clustering showed that *CLDN-1* expression was closely clustered with *IL-31* but loosely clustered with *L-4*, *IL-10*, *IL-13*, and *IL-17A*. We suggest that the alteration of claudin-1 in atopic dogs may disrupt the skin barrier and allow an influx of allergens, inciting inflammatory cytokine responses.

Key words: Canine atopic dermatitis, claudin-1, cytokine, tight junction

1. Introduction

Canine atopic dermatitis is the most common inflammatory skin disease of dogs (1,2). Many features of this disease are associated with skin barrier dysfunctions of the epidermis that are also seen in human atopic dermatitis (1,3,4). Epidermal composition defects of cross-linked cornified envelope (CE) proteins, such as filaggrin, involucrin, and loricrin, can impair skin functions in humans and dogs with atopic dermatitis (4,5). However, several studies have shown that skin barrier components found in the stratum corneum (SC) might have important roles in this defect (6,7). Tight junctions (TJs), also known as 'cross-bridged intercellular contacts', are the type of junctional complex located between two neighboring epithelial cells. It has been documented that many pathological skin conditions are associated with the changes of TJ morphology and function (8–10).

Claudin-1, one of the members of TJs present in all layers of epidermis, is encoded by the *CLDN-1* gene. It is a crucial component of the epidermal morphology of skin (6,8,10). In mice (8) and humans (10), the expression of claudin-1 in the epidermis has been seen to be concentrated in the cell borders of keratinocyte of the stratum granulosum (SG) to the stratum basale. In dogs, claudin-1 is present in all epidermal layers, except the SC (11), whereas in the SG of canine footpads, its expression is low (12). Dysfunction of claudin-1 can lead to weak skin barrier integrity as observed in wrinkled mice (8), dogs (13), and humans with atopic dermatitis (14). In atopic skin, the protease activity of house dust mite allergen (Der p1) cleaves and disrupts claudin-1 (15). Furthermore, defect of claudin-1 can increase epidermal water loss from the skin and make it more susceptible to microbial invasion (16). Recent research suggests that compromised epidermal TJ barriers are associated with infiltrated T cells in a cytokine-rich environment (15,16). Decreased claudin-1 expression in atopic humans is modulated by the expression of Th-2 cytokines and also regulated by proinflammatory cytokines in inflammatory skin conditions (9,14,16). However, regarding epidermal TJ barriers, in particular, for claudin-1, a correlation between the protein and mRNA expression levels and atopic dermatitis in dogs has not yet been established. Therefore, the aim of this study was to investigate the expression of claudin-1 in naturally atopic dog skin in association with the active T cell subsets and their related cytokines. The

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outcome of this study might provide new insight into pathological invasion via epidermal barrier dysfunction in canine atopic dermatitis.

2. Materials and methods

2.1. Animal samples

A total of 22 biopsied skin samples were collected from 12 dogs with canine atopic dermatitis (4 Siberian huskies, 3 Golden retrievers, 3 Labrador retrievers, and 2 mongrel dogs) and 10 normal dogs were used as a control group (4 Siberian huskies, 4 Golden retrievers, and 2 Labrador retrievers). None of the atopic dogs had any other related chronic or systemic skin disease at the time of collection. The atopic dogs naturally had atopic dermatitis with chronic pruritus diagnosed by clinical appearance according to Favrot's criteria (2). To rule out occurrence of other skin diseases, cytological examinations for bacterial, fungal, and yeast infections were carried out. Ectoparasitic infestations such as flea and sarcoptic mange were excluded. For final diagnosis, histological examination of skin tissue was employed to confirm or exclude other inflammatory or parasitic skin diseases such as demodicosis, cutaneous lymphoma, or sebaceous adenitis. Atopic dogs were also withdrawn from steroids or antibiotic drugs for at least 6 weeks prior to the sample collection. The lesions were assessed according to the CADESI-03 and CADLI scores, wherein CADESI-03 >23 and CADLI >60 were considered severe (1,17). A piece of 6 mm in diameter was taken from lesional and nonlesional skin by punch biopsy based on the clinical appearance of all atopic dogs. The samples for immunohistochemistry and gene expression were placed in 10% neutral buffer formalin and in RNAlater solution (Ambion, USA), respectively.

This study was approved by the Animal Ethics Committee of Kasetsart University (ACKU 02956). All specimens used in this study were collected with permission from dog owners with informed consent.

2.2. Immunohistochemistry

Tissue sections (3–5 µm) were placed on a positively charged glass slide (Thermo Scientific, USA) and then incubated in citrate buffer (pH 6.0) for antigen retrieval. Primary rabbit anticlaudin-1 antibody (Cell Marque, USA) was incubated for claudin-1 localization, and primary rabbit polyclonal anti-CD3 (1:1000) was used as the first antibody for double immunohistochemistry (Cell Marque). Mouse monoclonal anti-CD4 (1:200) (Leica Microsystems, UK) and mouse monoclonal anti-CD8 (1:200) (Cell Marque) were incubated separately for the second staining. Secondary HRP-conjugated goat antirabbit IgG antibody (Envision DAKO, Denmark) was used to develop the signals. Immunological reactions were then detected with diaminobenzidine chromogen (Invitrogen, UK) and an alkaline phosphatase-conjugated goat antimouse IgG antibody (Leica Microsystems) (1:1000). Vulcan Fast Red Chromogen (BioCare Medical, USA) was used for visualization.

The evaluation of claudin-1 was carried out with an Olympus FSX-100 microscope (Olympus, Japan) with 20 fields at 200× magnification. The cellular infiltration was counted per linear millimeter square of skin. Cell-D digital image analysis software (Olympus) was used to evaluate claudin-1 intensity as described previously (4). Statistical analyses were performed by using the Kruskal–Wallis test with post hoc Dunn's test for claudin-1 localization and one-way ANOVA for cellular infiltration (GraphPad Prism 5.0, USA). $P < 0.05$ was considered significant.

2.3. RNA extraction and qRT-PCR of *CLDN*-1 and cytokine expressions

Total RNA was extracted using TRIzol reagent (Invitrogen). After removal of DNAse, RNA samples (~1 µg) were reverse-transcribed in order to synthesize cDNA using a qPCRBIO cDNA synthesis kit (PCR Biosystems, UK). The mRNA expression levels of *CLDN-1* and cytokine genes were quantitatively measured by real-time quantitative PCR (qPCR) (C1000 Touch Thermal Cycler, Bio-Rad, USA) using qPCRBIO SyGreen Mix Lo-ROX (PCR Biosystems). Each gene was amplified using oligonucleotide primer sets, either used previously (3,18) or newly designed by using Primer3 (v.0.4.0) (Table 1). The new primer sets were designed based on dog sequences available in the GenBank database and subsequently tested against dog genomes (CanFam2.0, Sep 2011 assembly). Housekeeping genes *RPS19* and *RPLO* were used for normalization (18,19). All evaluations were done in triplicate. The qPCR data were statistically analyzed by REST software based on the geometric mean of the housekeeping genes (20). The patterns and similarity of gene expression among the samples were analyzed by MultiExperiment Viewer (MeV) software (version 4.8) (20). The normalized gene expression values were log_2-transformed before hierarchical clustering analysis. All statistical analyses were performed with a statistical significance level of $P < 0.05$.

3. Results

3.1. Claudin-1 localization in atopic and normal control skins

In immunohistochemistry analysis, the staining patterns and intensity scores of claudin-1 in the cytoplasmic membrane of all suprabasal layers appeared to be different in the normal control skins compared to both lesional and nonlesional atopic skins. Moreover, intensity scores of the normal control skins were significantly greater than those of lesional ($P < 0.05$) and nonlesional ($P < 0.01$) skins (Figure 1; Table 2). In atopic skin, as deduced from the staining patterns, claudin-1 was significantly reduced

Table 1. Specific primers for transcript genes and housekeeping genes.

Specific gene primer	Forward primer (5'-3')	Reverse primer (5'-3')	Accession number	Product size (bp)
IFN-γ	ccgactaggcaagactgtga	cagtggctcatcatgcagac	NM_001003174.1	177
TNF-α	actggagaagggtgatcgac	gtttgggcaagaatggacgt	NM_001003244.4	129
TGF-β1	atgttgtttttcggggacag	atgttgtttttcggggacag	NM_001003309.1	245
IL-4	cactcaccagcacctttgtc	aggtcttgtttgccatgctg	AF187322.1	264
IL-10	gctccaggagaaaggtgtct	agagagaggtatgacggggt	NM_001003077.1	230
IL-13	gtcttgctgtacctgggagt	gcagacaagagcagcatctc	NM_001003384.1	246
IL-17A	tacccctctgtgatctggga	catggcgaacaataggggtg	AB514445.1	208
IL-31	cctgttcctgctctgctcta	tgagacacagcagcaaggta	NM_001165914.1	188
CLDN-1	catggcgaacaataggggtg	atgttgtttttcggggacag	XM_845155.2	284
RPS19	ccttcctcaaaaagtctggg	gttctcatcgtagggagcaag	XM_533657	95
RPLO	ttgtggctgctgctcctgtg	atcctcgtccgattcctccg	XM_003433128.1	107

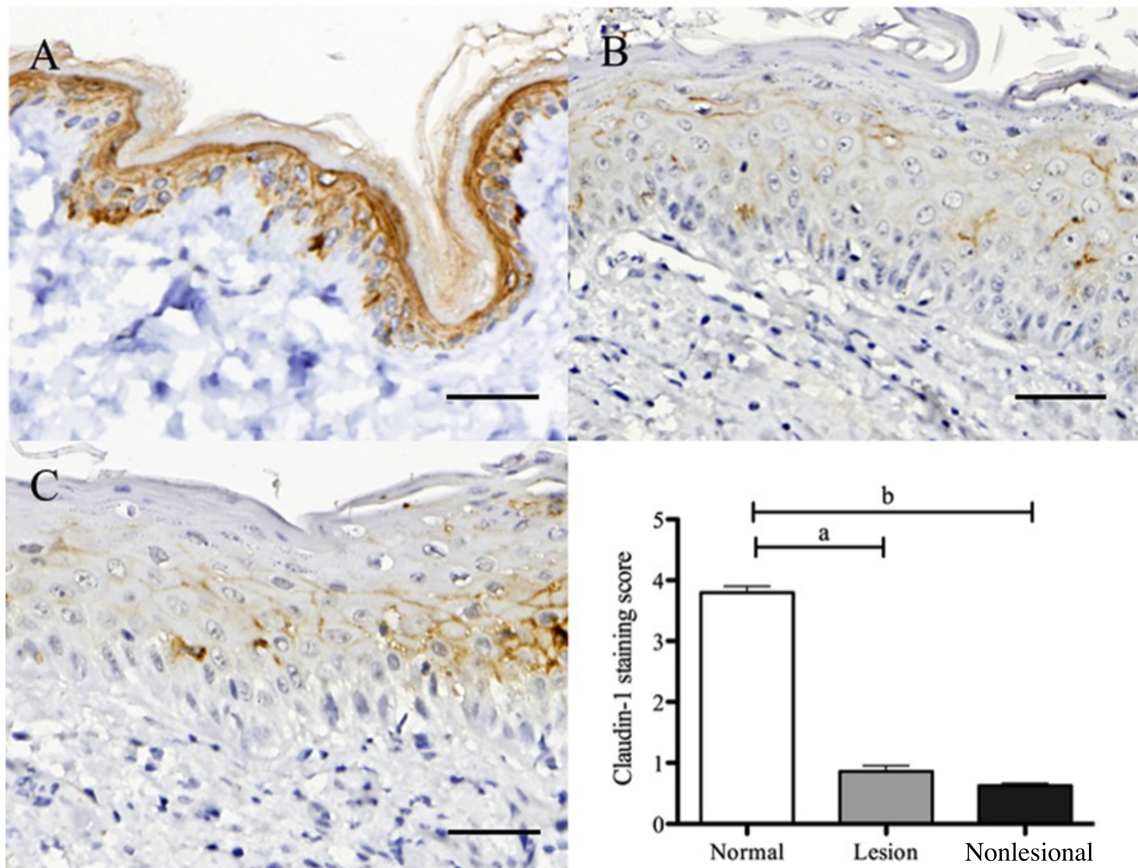

Figure 1. Claudin-1 localization in the biopsied skins: normal skin (A) and the lesional (B) and nonlesional (C) atopic dog skins (immunohistochemical staining, bar = 50 µm). Claudin-1 intensity score is given as mean ± SEM (a = $P < 0.05$, b = $P < 0.01$).

Table 2. Inflammatory infiltrating cells and claudin-1 intensity scores in the atopic and normal control skins.

Group	CD3+/CD4+ T cells	CD3+/CD8+ T cells	Claudin-1 staining score	Neutrophils (cells/mm^2)	Eosinophils (cells/mm^2)
All atopic dogs (n = 12)	403.01 ± 153.10^c	176.9 ± 91.85^c	0.86 ± 0.30^a	43.52 ± 21.54^c	54.05 ± 42.19^b
Atopic dogs with CADESI <120 and CADLI <23 (n = 6)	393.40 ± 95.73^a	174.30 ± 70.46^b	0.84 ± 0.30^a	37.73 ± 16.12^c	47.42 ± 39.50^b
Atopic dogs with CADESI >120 and CADLI >23 (n = 6)	438.10 ± 147.10^b	172.00 ± 67.85^b	0.88 ± 0.34^a	49.32 ± 27.07^c	60.67 ± 49.76^c
Normal control dogs (n = 10)	12.57 ± 5.31	10.02 ± 4.89	3.79 ± 0.32	1.64 ± 1.94	0.84 ± 1.18

The values are presented as mean ± SEM. Values within a column followed by different letters are significantly different when compared with those of the normal control skins (^{a} = P < 0.05, ^{b} = P < 0.01, ^{c} = P < 0.001).

in the cytoplasmic membrane of the SG and the stratum spinosum. A discontinuous staining pattern, particularly in the area of parakeratotic and spongiotic lesions, was found. However, there was no significant difference in the staining score of claudin-1 between the lesional and the nonlesional skins or between the atopic dogs with a higher clinical score (CADESI-03 >120; CADLI >60) and those with a lower clinical score (CADESI-03 <120; CADLI <60) (P > 0.05).

3.2. Cellular infiltration in the canine atopic dermatitis and normal control skins

Populations of inflammatory infiltrating cells (neutrophils, eosinophils, T cell subpopulations of CD3+/CD4+ T cells, and CD3+/CD8+ T cell subsets) were determined (Figure 2; Table 2). The CD3+/CD4+ and CD3+/CD8+ T cell numbers were significantly increased in lesional and nonlesional skins compared to the normal control group with P < 0.01 and P < 0.05, respectively. It should be noted that the numbers of the CD3+/CD4+ T cell sub-subset were significantly greater than those of the CD3+/CD8+ T cell sub-subset in atopic skin. The neutrophil and eosinophil numbers increased and were occasionally accompanied by microaggregation in the lesional skin (data not shown).

3.3. mRNA expression and hierarchical clustering analysis

The expression level of the *CLDN-1* gene in lesional skins was significantly lower than in normal skins (P = 0.032) with a 0.033-fold change indicating a marked downregulation of the gene in the lesional skins. In addition, the expression levels of *IL-4*, *IL-17A*, and *IL-31* were significantly greater in the lesional skins than in normal control skins with 4.932-fold (P = 0.002), 6.644-fold (P = 6.644), and 10.525-fold (P = 10.525) changes, respectively. TNF-α and IL-17 in the nonlesional skins were significantly higher than in normal skins with 4.324-fold (P = 0.042) and 9.243-fold (P = 0.020) changes. There were no significant differences between the lesional and nonlesional skins. These results indicated that the *CLDN-1* gene is downregulated in atopic dermatitis, especially in lesional skins. This was confirmed by the increase in some of the cytokines of the proinflammatory T cells (Table 3).

Hierarchical clustering analysis showed that there were two main clusters of the gene expression pattern (Figure 3): 1) *TNF-α*, *TGF-β1*, and *IFN-γ*, and 2) *IL-4*, *IL-10*, *IL-13*, *IL-3*, *IL-17A*, and *CLDN-1*. This indicates that a significant degree of mRNA coexpression was observable, particularly in this condition. Moreover, *CLDN-1*, a major gene associated with TJs, was more closely related to *IL-31* than other cytokines within the same subcluster.

4. Discussion

This study is the first report characterizing the expression of claudin-1 within TJs in dogs with atopic dermatitis by measuring the presence of claudin-1 protein and *CLDN-1* gene expression. Immunohistochemistry indicated that the location of claudin-1 in the normal dog skins was similar to that of healthy skins of humans (6), mice (8), and other dogs (11). The staining score of claudin-1 was significantly reduced in both lesional and nonlesional skins compared to the normal control. This observation was confirmed by measuring the gene expression level of *CLDN-1* in each of the dogs studied. Our results indicate that the claudin-1 protein corresponds to the first skin barrier protection, similar to earlier reports in dogs (13) and humans (14–16). It should be noted that allergens could easily penetrate into deeper epidermal layers due to defects in the first skin barrier at low levels of SC and CE proteins (7,14,15). In addition, the expression of CE proteins was significantly reduced in atopic dogs (4). However, an alternation in SC lipid composition and filaggrin protein in humans with

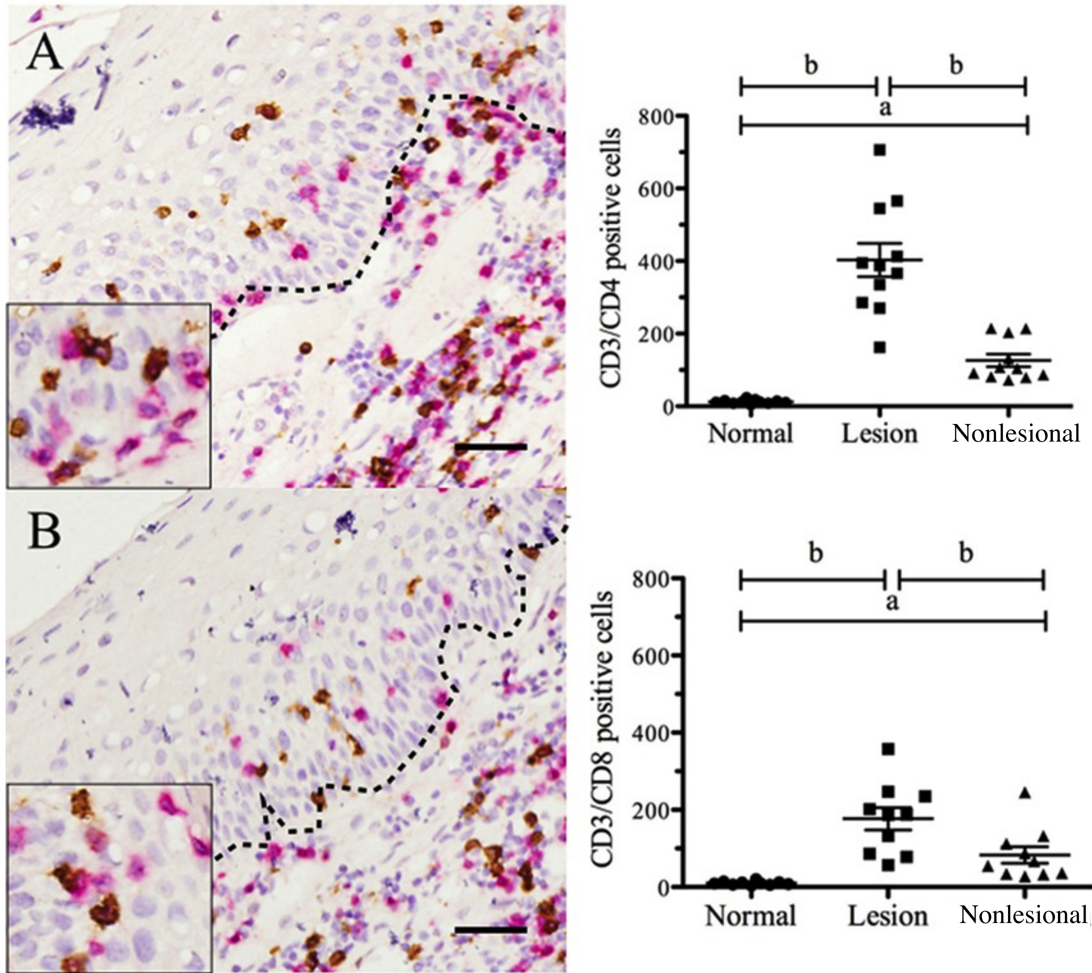

Figure 2. Double immunohistochemistry of CD3+/CD4+ (A) and CD3+/CD8+ T cell subsets (B) demonstrating infiltration into the epidermis and diffused aggregation in the dermis. Colocalization of all positive T cells is presented in the insets (CD3+ in brown color and CD4+, CD8+ in red color, double immunohistochemical stain, bar = 50 µm). Numbers of each T cell subset are compared between biopsied samples (a = $P < 0.05$, b = $P < 0.01$).

Table 3. Fold change in specific mRNA expressions in the atopic and normal control skins.

Gene	Lesional atopic vs. normal		Nonlesional atopic vs. normal		Lesional vs. nonlesional atopic	
	Fold change	P-values	Fold change	P-values	Fold change	P-values
IFN-γ	1.422	NS	1.662	NS	0.855	NS
TNF-α	2.462	NS	4.324	**0.042**	0.569	NS
TGF-β1	0.002	NS	0.692	NS	0.003	NS
IL-4	4.932	**0.002**	1.923	NS	2.554	NS
IL-10	0.775	NS	0.735	NS	1.053	NS
IL-13	1.507	NS	0.554	NS	2.720	NS
IL-17A	6.644	**0.002**	9.243	**0.020**	0.718	NS
IL-31	10.525	**0.001**	1.834	NS	5.740	NS
CLDN-1	0.033	**0.032**	0.582	NS	0.572	NS

A pair-wise fixed reallocation test (REST) was normalized by the geometric mean of two housekeeping genes, *RPS19* and *RPLO*. Values in bold indicate significant difference at $\alpha = 0.05$. NS = nonsignificant.

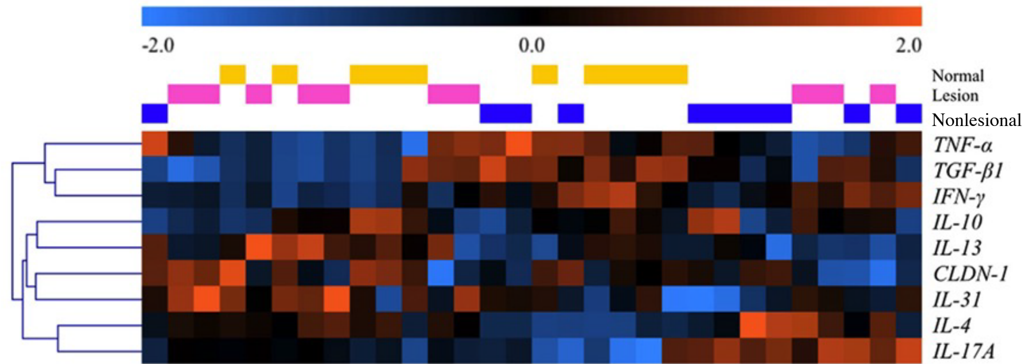

Figure 3. Hierarchical clustering for grouping of mRNA expressions of the biopsied samples: *CLDN-1* = claudin-1, *IFN-γ* = interferon-gamma, *IL-4* = interleukin-4, *IL-10* = interleukin-10, *IL-17A* = interleukin-17A, *IL-31* = interleukin-31, *TNF-α* = tumor necrotic factor-alpha, and *TGF-β1* = transforming growth factor-beta 1.

atopy and psoriasis has also been reported (7,10). Several studies have documented that deficiency of claudin-1 is associated with epidermal water loss in mice (8). Decreased claudin-1 protein expression has been recently reported in atopic dogs, but the other TJs, such as ZO-1 and occludin, did not show any association (13). Moreover, in contrast to earlier studies, the mRNA expression level of *CLDN-1* was not significant in small-breed dogs (3). One possible reason is that the TJ expression in genetic-associated diseases might be more clearly noticed in purebred dogs. Further study is required with a larger sample size to confirm these results.

In this study, numbers of CD3+/CD4+ T cells were significantly greater than numbers of CD3+/CD8+ T cells in atopic skin. This could be explained by the presence of eosinophils and Th1/Th2-producing cells in response to the inflammatory response (21). This relatively high Th2 cell number was also observed in pruritic dogs with increased *IL-4* mRNA level (22). As an effect of immunological response, increased levels of some cytokines, e.g., IL-4, IL-17A, and IL-31, associated with the depletion of CLDN-1 in atopic skin, were also found. Filaggrin expression can be downregulated by IL-7 stimulation in keratinocytes. Moreover, the effect of this IL-7 has been shown in reducing transcription of TJ proteins including the claudin family, ZO-1, plankoglobins, plakophilins, and cadherins (23,24). This suggests that IL-17A-dominated cytokine expression in atopic skin disease can promote skin barrier dysfunction. We found that neutrophil aggregation in the nonlesional skins might be associated with the synergistic effects of IL-17A and TNF-α on continued inflammation and neutrophil recruitment (25). The upregulation of TJ requires TNF-α to increase transepidermal resistance in the early stage of psoriatic skin (9). Therefore, the effect of TNF-α alone could have indirectly affected *CLDN-1* mRNA levels in the nonlesional skins in our study. Several studies reported increasing levels of IFN-γ and IL-10 in chronic atopic dermatitis in humans (25,26). However, we could not see any difference in these two parameters between dogs with atopic dermatitis and normal dogs, as we considered that dogs with atopic dermatitis in this study were in the acute phase.

IL-31 mRNA level was significantly greater in the lesional atopic skins compared to the normal control, as shown in Table 3. The same results were reported in human atopic skins with higher levels of Th2 rather than Th1 cells (27,28) and were also observed in pruritic dogs (22,29). Therefore, IL-31 can be an important indicator of immunological response in atopic disease. According to the hierarchical clustering, *CLDN-1* expression can be used to confirm the relevance of several cytokines as the indicator of gene coexpression partners in atopic skin.

This is the first report showing a relationship between claudin-1 and inflammatory cytokines in atopic dog skins compared to normal skins. Our results suggest that claudin-1 may play an important role in the first skin barrier protection and could be used as a potential indicator for canine atopic dermatitis in the future. Further study with a larger sample size would be important to confirm the results presented in this study.

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