รายงานวิจัยฉบับสมบูรณ์

โครงการการศึกษายีนกลุ่มไทท์จังชั่นในโรคผิวหนังชนิดอะโทปีในสุนัข

โดย

กรรณาภรณ์ สุริยผล และ ปิยะรัตน์ จันทร์ศิริพรชัย

มิถุนายน 2557

สัญญาเลขที่ MRG5580144

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สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา และสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกอ. และ สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

โครงการนี้ได้รับอนุมัติเงินทุนพัฒนาศักยภาพในการทำงานวิจัยของอาจารย์รุ่นใหม่ตามโครงการความร่วมมือระหว่างสำนักงานคณะกรรมการอุดมศึกษาและสำนักงานกองทุนสนับสนุนการวิจัย ปีงบประมาณ 2555 คณะผู้วิจัยขอขอบคุณอาจารย์สัตวแพทย์หญิง ดร.ศิรินทร์ ธีระวัฒนศิริกุลและนายสัตวแพทย์ทศพร นักเบศ ที่ให้คำแนะนำและความช่วยเหลือในงานวิจัย

คณะผู้วิจัย
มิถุนายน 2557

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โรคผิวหนังชนิดอะโทปีหรือโรคผื่นภูมิแพ้ผิวหนังเป็นโรคที่ทำให้ผิวหนังอักเสบและคันที่พบได้บ่อยทั้งในคนและสุนัข โปรตีนกลุ่มรอยต่อระหว่างเซลล์ (เซลล์จังชัน) และกลุ่มคอร์นิไฟด์เอนเวลโลปมีความสำคัญในการสร้างและความอยู่ตัวของผิวหนัง วัตถุประสงค์ของการศึกษาครั้งนี้คือต้องการตรวจสอบการแสดงออกของยีนกลุ่มรอยต่อระหว่างเซลล์และยีนที่เกี่ยวข้องกับการสร้างคอร์นิไฟด์เอนเวลโลปในโรคผื่นภูมิแพ้ผิวหนังในสุนัขพันธุ์เล็ก โดยทำการตัดชิ้นเนื้อจากผิวหนังสุนัขที่มีรอยโรค 10 ตัว ไม่มีรอยโรค 9 ตัว เปรียบเทียบกับผิวหนังสุนัขปกติ 11 ตัว นำมาทำปฏิกิริยาลูกโซ่พอลิเมอเรสเรียลไทม์แบบย้อนกลับ ยีนกลุ่มรอยต่อระหว่างเซลล์ที่ได้ทำการศึกษาได้แก่ คลาวดิน-1 คลาวดิน-23 อ็อกคลูดิน โซนาอ็อกคลูเดนส์-1 และ -2 โซนาอ็อกคลูเดนส์-1 แอสโซสิเอดเต็ดนิวคลีอิกแอซิดบายดิงโปรตีน (*ZONAB*) ซินกูลิน แก็ปจังชันเบต้า-2 (*GJB2*) และอีแคทฮีริน และได้ทำการศึกษายีนทรานส์กลูตามิเนส-1 (*TGM1*) ซึ่งมีหน้าที่เชื่อมโปรตีนในกลุ่มคอร์นิไฟด์เอนเวลโลป นอกจากนี้ยังได้ทำการตรวจนับจำนวนเซลล์เม็ดเลือดขาวบนชิ้นเนื้อดังกล่าวและศึกษาความสัมพันธ์ของจำนวนเซลล์เม็ดเลือดขาวกับดัชนีแสดงความรุนแรงของโรค (CADESI-03) จากการศึกษาพบการแสดงออกของยีน *GJB2* และ *TGM1* เพิ่มขึ้นอย่างมีนัยสำคัญ ณ ผิวหนังบริเวณที่มีรอยโรค พบการแสดงออกของยีน *ZONAB* ลดลงอย่างมีนัยสำคัญ ณ ผิวหนังส่วนที่ไม่ใช่รอยโรค จากการจัดกลุ่มการแสดงออกของยีน *GJB2* และ *TGM1* แบบเป็นขั้นตอนพบลักษณะการแสดงออกของยีนดังกล่าวคล้ายคลึงกับยีนเคราตินหลายตัวซึ่งอาจแสดงถึงการแสดงออกร่วมกันในโรคผื่นภูมิแพ้ผิวหนัง นอกจากนี้ยังพบเซลล์เม็ดเลือดขาวประเภทนิวโทรฟิล โมโนไซต์ อีโอสิโนฟิลและมาสเซลล์เพิ่มขึ้นอย่างมีนัยสำคัญ ณ ผิวหนังบริเวณที่มีรอยโรค แต่ไม่พบความสัมพันธ์ของจำนวนเซลล์เม็ดเลือดขาวกับดัชนีแสดงความรุนแรงของโรค สรุปได้ว่าการศึกษาครั้งนี้ได้รายงานการแสดงออกของยีน *GJB2* และ *TGM1* ในโรคผื่นภูมิแพ้ผิวหนังในสุนัข และได้แสดงความสัมพันธ์ระหว่างรอยต่อระหว่างเซลล์และ ทรานส์กลูตามิเนส-1 กับโรคผื่นภูมิแพ้ผิวหนังในสุนัขเป็นครั้งแรก

คำสำคัญ: โรคผิวหนังชนิดอะโทปีในสุนัข แก็ปจังชั่นเบต้า 2 ทรานส์กลูตามิเนส 1 เซลล์จังชัน การแสดงออกของยีน เซลล์เม็ดเลือดขาว สุนัข

Abstract

Project Code : MRG5580144

Project Title : Study the tight junction gene expression in canine atopic dermatitis

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Atopic dermatitis is a common pruritic inflammatory skin disease in humans and dogs. Cell junction and cornified envelope are groups of proteins that are crucial for the formation and stability of the skin barrier. The purpose of this study was to investigate gene expression in cell junction and cornified envelope groups in canine atopic dermatitis (CAD) in small breed dogs. Skin biopsy was performed from 10 lesional CAD, 9 non-lesional CAD cases and 11 normal dogs and subjected to quantitative reverse transcription-polymerase chain reaction. Several cell junction genes were evaluated, including claudin-1, occludin, zonula occludens-1 and -2, zonula occludens-1-associated nucleic acid binding protein (*ZONAB*), cingulin, gap junction beta 2 (*GJB2*) and e-cadherin together with transglutaminase 1 (*TGM1*), a cross-linker of the cornified envelope. In addition, cell filtration at the site was examined. An upregulation of *GJB2* and *TGM1* were significantly observed in lesional skin. *ZONAB* was found to be downregulated in the non-lesional skin. Hierarchical clustering showed the similarities in patterns of gene expression of *TGM1*, *GJB2* and several cell proliferated and/or differentiated keratins which might indicate co-expression partners. The filtration of neutrophils, monocytes, eosionophils and mast cells to the site were significantly observed in lesional skin. However, no association of cell infiltration and canine atopic dermatitis extent and severity index (CADESI-03) was observed. In conclusion, the present study demonstrates the expression of *GJB2* and *TGM1* in CAD. This is the first report of an association of cell junction and *TGM1* genes with CAD.

Keywords: canine atopic dermatitis, gap junction beta 2, transglutaminase 1, cell junction, gene expression, white blood cell, dog

Executive Summary

Atopic dermatitis is a commonly allergic skin disease in humans and dogs and the defect of skin barrier has been shown to be associated with the disease. A cell junction or intercellular bridge is a contact of adjacent cells or between a cell and the extracellular matrix especially in epithelial tissues, including skin, contributing the paracellular barrier and helping the paracellular transport. In skin diseases which are characterized by impaired skin barrier function, altered proliferation/differentiation of the epidermis and/or infiltration of inflammatory cells, alterations of the expression patterns of cell junction genes have been described. The cornified envelope (CE) is a protein complex layer beneath the plasma membrane of mammalian epidermis in terminal differentiation of the skin. It helps protect skin cells against water loss and infection and maintain epidermal structure integrity. Gene expression of a number of CE proteins has been reported in canine atopic dermatitis (CAD). In the present study, we have investigated the association of the cell junction genes, including claudin-1 (*CLDN1*), claudin-23 (*CLDN23*), occluding (*OCLN*), zonula occludens-1 (*ZO-1*) and -2 (*ZO-2*), zonula occludens-1-associated nucleic acid binding protein (*ZONAB*), cingulin (*CGN*), gap junction beta 2 (*GJB2*) and e-cadherin (*CDH1*) together with transglutaminase 1 (*TGM1*), an enzyme that facilitates crosslinking of the CE proteins in mature keratinocytes. In addition, CAD-associated inflammatory cell infiltrate and its relationship with CADESI-03 scores were also characterized. From quantitative reverse transcription polymerase chain reaction (qRT-PCR), we found the upregulation of *GJB2* and *TGM1* in lesional skin. *ZONAB* was found to be downregulated in the non-lesional skin. Hierarchical clustering showed the similarities in patterns of gene expression of *TGM1*, *GJB2* and several cell proliferated and/or differentiated keratins which might indicate co-expression partners. From histology, the filtration of neutrophils, monocytes, eosionophils and mast cells to the site were significantly observed in lesional skin. However, no association of cell infiltration and canine atopic dermatitis extent and severity index (CADESI-03) was found. Since canine epidermis behaves very much like human and mouse epidermis with regard to changes in *GJB2* expression associated with keratinocyte hyperproliferation, this congruence supports the use of dogs as experimental models to study human skin pathologies. Taken together, these studies establish an intriguing correlation between increased *GJB2* and *TGM1* expression and CAD. For the future work, the clinical application of TGM1 and GJB2 expression in CAD should be investigated.

Table of Contents

List of Tables

List of Figures

1. Introduction

Atopic dermatitis (AD) or atopic eczema is the common allergic skin disease recognized in dogs and human (Hillier and Griffin, 2001; Wadonda-Kabondo et al. 2003). Canine atopic dermatitis (CAD) is originally defined as “genetically predisposed inflammatory and pruritic allergic skin disease with characteristic clinical features associated with IgE antibodies most commonly directed against environmental allergens” (Halliwell, 2006).

The epidermal barrier is important to prevent allergens from penetrating the skin. Defects in the skin barrier functions lead to increased access of allergens to antigen presenting cells in subepithelial tissues and triggering hypersensitivity and allergic reaction in dogs with AD (Marsella et al., 2011). A cell junction or intercellular bridge is a contact of adjacent cells or between a cell and the extracellular matrix especially in epithelial tissues, contributing the paracellular barrier and helping the paracellular transport. In vertebrate, cell junctions can be functionally classified into 4 types, including i.) anchoring junctions ii.) gap or communicating or channel-forming junctions, iii.) tight or occluding junctions, and iv.) signal-relaying junctions (Alberts et al., 2007). Anchoring-type junctions function to hold cells and provide strong structural cohesion between adjacent cells in tissues that have to stand constant mechanical stress e.g. skin and cardiac muscle (Kuwahara et al., 2001; Mezzano and Sheikh, 2012). Protein members in this group include cadherins (CDH), catenins, integrins, etc. Gap junctions are intercellular channels that allow communication of small molecules (metabolites, second messengers, and ions), up to a molecular weight of about 1000 daltons between cells (Sosinsky and Perkins, 2000), leading to cell proliferation, cell death, tumor suppression, and action potential in several organs such as heart, brain, retina, and skin (Becker et al., 2002; Clarke et al., 2006; Kretz et al., 2004; Teubner et al., 2001).There are several gap junction proteins such as gap junction alpha 1 protein (GJA1) or connexin 43 (Cx43) and gap junction beta 2 protein (GJB2) or Cx26 (Bruzzone, 2001). Tight junctions (TJs) are intercellular junction that adhere two neighboring cells near the apical side of cells. A number of TJ proteins have been identified in mammalian epidermis including occludin (OCL), cingulin (CGN), zonula occludens-proteins (ZOs), claudins (CLDNs), and junctional adhesion molecules (JAMs) (Kirschner et al., 2010). CGN can form complexes with ZO-1, ZO-2 and JAM-1 (Guillemot and Citi, 2006). In skin diseases which are characterized by impaired skin barrier function, altered proliferation/differentiation of the epidermis and/or infiltration of inflammatory cells, alterations of the expression patterns of TJ genes were described such as the downregulation of *CLDN1*, *CLDN3*,

and *JAM-1* and upregulation of *ZO-1* and *OCLN* in psoriasis (Kirschner et al., 2009; Watson et al., 2007). Upregulation of ZO-1 and OCLN proteins in early stage of *Staphylococcus aureus* infection and upregulation of CLDN-1 and OCLN proteins in mouse skin exposed to UV radiation were also shown (Ohnemus et al., 2008). In atopic dermatitis, CLDN1 and CLDN23 protein expression were decreased in HAD patients whereas GJB2 was inversely upregulated (De Benedetto et al., 2012). The association of TJ gene expression with CAD has not yet been demonstrated.

The cornified envelope (CE) is a protein complex layer beneath the plasma membrane of mammalian epidermis in terminal differentiation of the skin. It helps protect skin cells against water loss and infection and maintain epidermal structure integrity (Hohl, 1990; Reichert et al., 1993). Gene expression of a number of CE proteins, including involucrin (IVL), filaggrin (FLG) together with other epidermal differentiation and proliferation markers such as keratin 5 (KRT5) and KRT 10 have been reported in normal dog skin in different breeds and coat types (Theerawatanasirikul et al., 2012[a]) and in CAD (Theerawatanasirikul et al., 2012[b]; Theerawatanasirikul et al., 2012[c]). Transglutaminase 1 (TGM1) is an enzyme that facilitates cross-linking of the CE proteins in mature keratinocytes by catalyzing formation of Ɛ-(γ-glutamyl)-lysine cross-links in proteins (Greenberg et al., 1991). However, the association of TGM1 and CAD has not yet been demonstrated. In addition, the inflammatory cell filtration at the site and its relationship with the Canine Atopic Dermatitis Extent and Severity Index (CADESI-03) were also characterized. The objective of this study, therefore, was to characterize the gene expression patterns of *CGN*, *CLDN1*, *CLDN23*, *CDH1*, *GJB2*, *OCLN*, *ZO-1*, *ZO-2*, *ZONAB* and *TGM1* genes in lesional atopic, non-lesional atopic and healthy canine skin by the quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The study would give better understanding of the CAD disease.

2. Objectives

To study *CGN*, *CLDN1*, *CLDN23*, *CDH1*, *GJB2*, *OCLN*, *ZO-1*, *ZO-2*, *ZONAB* and *TGM1* mRNA expression from epidermal keratinocyte of dogs with atopic dermatitis and normal dogs.

3. Materials and Methods

Animals

Nineteen atopic dogs from private small animal clinics, fulfilled at least 5 signs of the diagnostic criteria for CAD (Favrot et al., 2010; Olivry 2010), including onset of signs under 3 years

of age, dog living mostly indoors, glucocorticoid-responsive pruritus, pruritus sine materia at onset, affected front feet and/or ear pinnae, and nonaffected ear margins and/or dorso-lumbar area. This group comprised of eleven Poodles, six Shih tzus and two Pugs, with a mean age of seven years (age range 2–11 years). Clinical lesions of CAD were scored using CADESI-03. The third version of the CADESI (CADESI-03) scale consists of the evaluation of 4 different lesions (erythema and excoriations for acute stage, and lichenification and self induced alopecia for chronic stage) at 62 body sites with a severity scale ranging from 0 to 5 as follows: none (0), mild (1), moderate (2,3), and severe (4,5). Hence, the maximal achievable score was 62x4x5 = 1240 (Olivry et al., 2007). The total score from all clinical signs and body sites was statistically analyzed. Eleven healthy control dogs comprised of seven Poodles, three Shih tzus and one Pug, with a mean age of seven years (age range 1–10 years).

Skin biopsies and tissue samples

Skin specimens were all taken from the ventral area of each dog to minimize variations due to body location. Lesional samples of erythematous, macular-papular dermatitis and lichenification were selected from the affected areas. Non-lesional samples were obtained from the clinically unaffected skin of atopic dogs whereas control samples were from clinically normal dogs. Punch skin biopsies (6 mm) were obtained after local anesthesia with 2% lidocaine and, then, sutured routinely. Subcutaneous fat was stripped off before each biopsy was bisected. One half was immersion fixed in 10% neutral buffered formalin for 24 h, followed by standard tissue processing and paraffin embedding for a routine histopathological study. The other half was kept in RNALater solution (Life Technologies, Carlsbad, CA) overnight at 4^{o}C and stored at -20^{o}C until being processed for quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). The sample collection and processing procedures were approved by the Chulalongkorn University Animal Care and Use Committee (CU-ACUC), Thailand.

RNA Extraction

The skin tissues in RNALater solution were disrupted in liquid nitrogen to maintain a low temperature. Total RNA was extracted from the skin tissues by homogenization with Trizol reagent (Life Technologies, Carlsbad, CA) and phenol/chloroform/isopropyl alcohol. Subsequently, genomic DNA traces were removed from the RNA with Turbo DNase (Ambion, Austin, TX) to purify the total RNA according to the instructions. The DNase-treated RNA quality and concentration were analyzed using a NanoDrop ND-1000 Spectrophotometer V3.7 (Thermo Fisher Scientific, Waltham,

MA).

Quantitative reverse transcriptase PCR

The SuperScript III First-strand synthesis system for RT-PCR (Life Technologies, Carlsbad, CA) was used to synthesize cDNA according to the manufacturer's instructions. Briefly, one microgram of RNA was reverse transcribed in a 20 µL reaction containing 50 ng random primers, 40U RNase inhibitor and 200U Superscript III enzyme. The Rotor Gene 3000 Thermal Cycler (Qiagen, Hilden, Germany) was used to perform quantitative PCR. Except for the housekeeping gene (HKG), RPS19, which has been previously described as a suitable HKG for CAD (Schlotter et al., 2009; Theerawatanasirikul et al., 2012[c]), all primers were designed by the Primer 3 program version 0.4.0 (http://frodo.wi.mit.edu/). Primer pairs were sequenced for specificity and uniqueness in the dog genome (CanFam 2.0, May 2005 assembly). The primers sequences, melting temperatures and amplicons are depicted in Table 1.

Table 1 Primers used in the present study. Indicated are sequences, annealing temperatures in real-time PCR reactions and expected product sizes.

Genes	Primers (5'to 3')	Amplicon (bp)	Accession number
CGN	Fwd 5'-agctcggatgaggagtttga-3'	277	DQ910799
	Rev 5'-agaggcaagcctgtctacca-3'		
CLDN1	Fwd 5'-ggccactattggcatgaagt-3'	284	XM845155.2
	Rev 5'-atgttgtttttcggggacag-3'		
CLDN23	Fwd 5'-gtggacgtggagctgtacc-3'	293	XM003639565.1
	Rev 5'-cggtgggtgtaccaggac-3'		
CDH1	Fwd 5'-ggtgctcacatttcccagtt -3'	100	NM001197148.1
	Rev 5'-aaatgggcctttctcgtttt-3'		
GJB2	Fwd 5'-aaatatgggccggatagacc-3'	180	NM001197148.1
	Rev 5'-tcccaagcaagctccttaaa-3'		
OCLN	Fwd 5'-catggtgattgtggcttttg-3'	180	NM001003195.1
	Rev 5'-ggaggaggcatgtcttgtgt -3'		
ZO-1	Fwd 5'-cggtaccagctcctctcttg-3'	153	NM001003140.1
	Rev 5'-cggtttggtggtctgaaagt-3'		

Table 1 Continued

ZO-2	Fwd 5'-caattcagcatcagcaagga-3'	198	NM001003204.1
	Rev 5'-gctcatccagctcattgtca-3'		
ZONAB	Fwd 5'-cggttcatcgaaatccaact-3'	234	AF171061.1
	Rev 5'-atggaacttcaggtgccttg-3'		
TGM1	Fwd 5'-gcaagaaggaagtggtgctc -3'	167	AF262219.1
	Rev 5'-acggaaggtatgctgtttgg-3'		
RPS19	Fwd 5'-ccttcctcaaaaa/gtctggg-3'	95	XM533657
	Rev 5'-gttctcatcgtagggagcaag-3'		

PCR reactions were performed in a 10 µL volume containing 1x KAPA SYBR Fast qPCR Master Mix Universal (KAPA Biosystems, Cambridge, MA), 200 nM of each primer and the cDNA template. Thermal cycling conditions were as follows: 95 ^{o}C for 2 min for one cycle followed by 40 cycles at 95 ^{o}C for 3 s, 60 ^{o}C for 20 s and 72 ^{o}C for 1 s. Each reaction was performed in duplicate in 3 independent runs. Data from the FAM/SYBR channel operating at an excitation maximum 495 nm and an emission maximum 520 nm was evaluated. A melting curve analysis was used to determine the purity of the amplified products. Relative expression levels were analyzed by the REST-384 (Relative Expression Software Tool) software. Standard curves were generated for each assay as previously demonstrated and threshold cycles of all targets in test samples were normalized to the corresponding *RPS19* levels in control samples (Pfaffl, 2001; Pfaffl et al., 2002; Theerawatanasirikul et al., 2012[c]).

Histology

FFPE sections of 3 microns were placed on glass slides for routine staining with hematoxylin and eosin (HE), toluidine blue, and chromotrope 2R. FFPE tissue was deparaffinized with xylene and rehydrated with a series of graded ethanols. After HE staining, cellular counting was performed by light microscopy. Ten fields were randomly selected at x200 magnification. By random in each section, the number of mononuclear cells, neutrophils, eosinophils and mast cells per unit area (mm^2) in the epidermis of lesion and non-lesion sections were counted compared with the normal controls, using the computerized image analyzer (Image-Pro® PLUS 6.0 Programming software, Media Cybernetics, Inc., USA.).

Statistic analysis

The data was analyzed in REST 384 software (Relative Expression Software Tool), using a pair wise fixed reallocation randomization test to test for significance between groups. Results with a p value <0.05 were considered significant. Standard curves will be generated for each assay using the fluorescent data from 10-fold serial dilutions of total RNA of the same normal dog. The PCR efficiency will be calculated from standard curve slope by a modified delta delta threshold cycle (C_T) method and expressed as target gene normalized to a reference gene. Hierarchical clustering was performed to analyze the pattern of similarity in gene expression in lesional skin in order to identify gene clusters, using Multiexperiment Viewer (MeV) program, version 4.8 (Saeed et al., 2003; Saeed et al., 2006). Statistical analysis of inflammatory cell infiltration and the correlation of determination (R^2) was conducted by the Pearson Correlation coefficient, using GraphPad Prism software, version 5.0 (GraphPad Software, La Jolla, CA). Statistical differences among multiple groups compared with a control will be determined using One-way Analysis of Variance (One-way ANOVA), and significant differences will be determined by a Tukey-Kramer test. Results with a p value < 0.05 will be considered of relevant significance.

4. Results

For qRT-PCR experiment, GJB2 and TGM1 expressed up-regulation in lesional skin compared to normal skin with fold changes 8.081 ($p = 0.007$) and 3.859 ($p = 0.038$), respectively). GJB2 also showed up-regulation in lesional skin compared to non- lesional skin with fold change 5.757 ($p = 0.025$). Lower expression of ZONAB was observed in non-lesional skin compared to normal skin (Table 2) with fold change 0.379 ($p = 0.003$). For hierarchical clustering in lesional skin, when compared with gene expression patterns of *FLG*, *IVL*, other *KRTs* in a previous report (Theerawatanasirikul et al., 2012[c]), *GJB2*, *TGM1*, and *KRT14* were clustered together in one sub-cluster and *KRT1*, *KRT5* and *KRT17* in the other. However both were placed in the same super-cluster with *IVL* and *FLG* whereas *KRT10* and *KRT2e* were clearly different (Fig. 1).

For cell infiltration, the inflammatory cell infiltration in lesional (acute, chronic, and total), non-lesional and normal skin was shown in Table 3. The cell infiltration in acute skin lesion, chronic skin lesion and non-lesional skin was compared with clinical severity scores CADESI-03. Only the eosinophil infiltration in chronic lesion was shown to be significantly negatively correlated with clinical severity scores ($p < 0.05$, $R^2 = 0.94$) (Fig. 2). For others, no significant correlation was found (Table 4).

Table 2 Fold change in gene-specific mRNA expression in CAD lesional, CAD non-lesional and control dog skin compared to RPS19 expression

Gene	lesion vs. normal		non-lesion vs. normal		lesion vs. non-lesion	
	Fold change	p value*	Fold change	p value*	Fold change	p value*
CGN	0.711	0.653	1.927	0.314	0.431	0.261
CLDN1	1.330	0.664	2.077	0.178	0.640	0.534
CLDN23	0.665	0.693	0.704	0.632	0.946	0.954
CDH1	1.739	0.363	0.889	0.756	1.955	0.261
GJB2	8.081**	0.007	1.404	0.531	5.757**	0.025
OCLN	0.895	0.883	1.278	0.537	0.700	0.652
ZO-1	8.397	0.499	88.364	0.219	0.095	0.440
ZO-2	1.747	0.367	1.745	0.175	1.001	0.998
ZONAB	0.503	0.333	0.379**	0.003	1.327	0.709
TGM1	3.859**	0.038	2.342	0.073	1.648	0.439

*Pair wise fixed reallocation randomization test normalized by reference gene

**Significantly different at $p < 0.05$

Fig. 1. Hierarchical clustering of genes in lesional skin. d = dog.

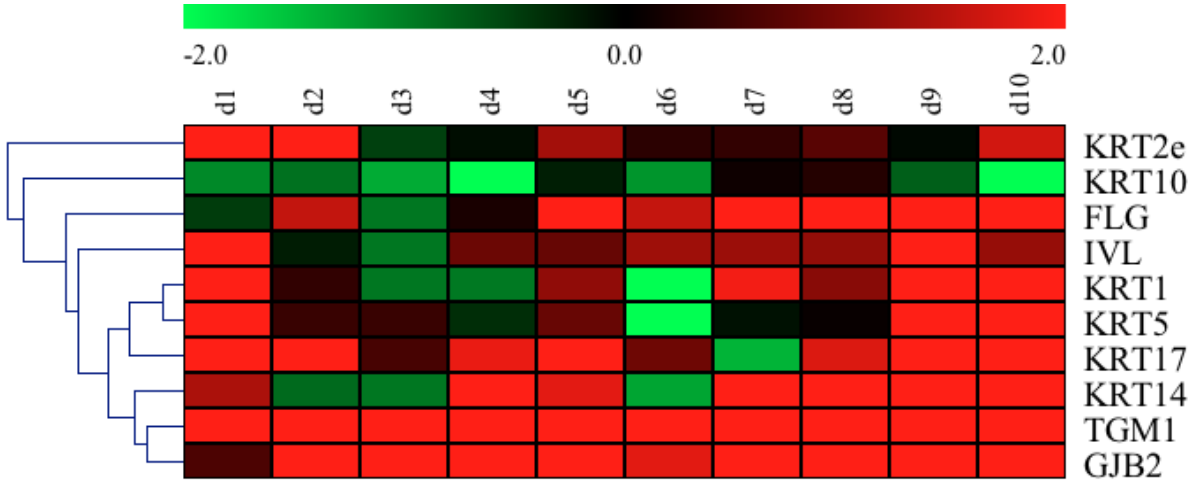

Table 3 The cutaneous inflammatory cell infiltration in canine skin

Cells (cells/mm^2)	Canine skin			
	Normal (n=11)	Non-lesion (n=9)	Acute lesion (n=6)	Chronic lesion (n=4)
Neutrophils	0.85±1.40	11.44±5.78	65.01±35.69[b,d]	53.92±40.33[a,c]
Mononuclear cells	17.50±14.62	51.48±23.67	277.88±123.44[e,f]	148.27±106.37
Eosinophils	0.31±0.54	15.18±10.23	49.03±29.28[h,j]	39.44±17.69[g,i]
Mast cells	20.21±12.15	48.24±15.51	88.41±23.75[k,l]	60.29±10.99

(a, b) Significantly different from normal at $p < 0.01$ (a) and $p < 0.001$ (b), (c, d) Significantly different from non-lesion at $p < 0.05$ (c) and $p < 0.001$ (d), (e, f) Significantly different from normal (e) and non-lesion (f) at $p < 0.001$, (g, h) Significantly different from normal at $p < 0.01$ (g) and $p < 0.001$ (h), (i, j) Significantly different from non-lesion at $p < 0.05$ (i) and $p < 0.001$ (j), (k) Significantly different from normal at $p < 0.001$, (l) Significantly different from non-lesion at $p < 0.01$.

Fig. 2. Correlation between CADESI-03 and eosinophil infiltration in chronic skin lesion of dogs with AD.

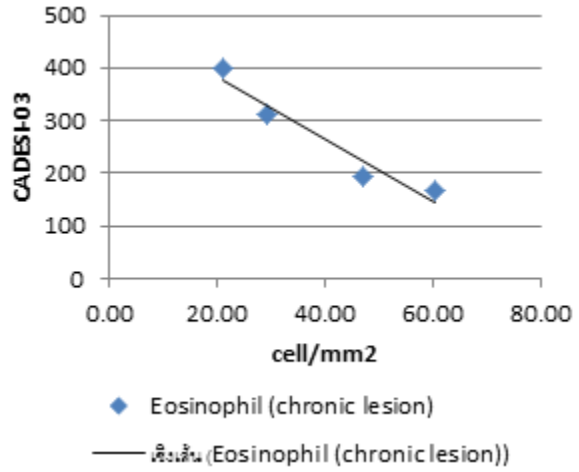

Table 4 Correlation between CADESI-03 and PMN, monocyte, eosinophil and mast cell infiltration in acute and chronic skin lesion of dogs with AD.

Dog No.	Acute or chronic lesion	CADESI-03	PMN	Monocyte	Eosinophil	Mast cell
1.	Acute	165	53.40	466.50	69.20	79.72
2.	Acute	269	48.00	340.80	90.56	90.67
3.	Acute	167	48.67	100.89	60.44	73.33
4.	Acute	357	31.50	333.33	25.92	66.94
5.	Acute	311	34.00	290.67	29.34	64.11
6.	Acute	220	115.55	140.21	9.28	132.94
7.	Chronic	167	48.67	100.89	60.44	73.33
8.	Chronic	357	31.50	333.33	25.92	66.94
9.	Chronic	311	34.00	290.67	29.34	64.11
10.	Chronic	220	115.55	140.21	9.28	132.94
R^2	Acute		0.4238	0.0527	0.0454	0.0014
R^2	Chronic		0.4673	0.0126	0.9386[a]	0.0498

[a] significantly correlation ($p < 0.05$)

5. Discussion

In the present study, we demonstrate the gene upregulation of GJB2 and TGM1 in CAD and this is the first report of an association of cell junction and transglutaminase 1 genes with CAD. Gap junction-mediated cell communication plays a role in maintaining a uniform epidermal thickness with the balance of cell proliferation and differentiation. Several reports showed the association of an increase in the number of keratinocytes and/or the thickening of the epidermis with the induction of GJB2 expression either at the mRNA or protein levels, including a lesion of human skin treated with retinoic acid (Masgrau-Peya et al., 1997), human psoriatic lesions (Hivnor et al., 2004; Labarthe et al., 1998; Lucke et al., 1999; Shaker and Abdel-Halim, 2012), human porokeratosis (Hivnor et al., 2004), tape-stripped epidermis and viral warts (Lucke et al., 1999). In mouse epidermis, GJB2 expression rapidly increased in hyperproliferative wound epidermis

(Djalilian et al., 2006; Goliger and Paul, 1995). In mouse skin papillomas, expression of both GJB2 and GJA1 was elevated in the proliferating neoplasms (Sawey et al., 1996). GJB2 was also upregulated in HAD (De Benedetto et al., 2012). In CAD, the hyperproliferation and aberrant keratinocyte differentiation resulted in the thickening of the epidermis (Theerawatanasirikul et al., 2012b). Since the hierarchical clustering showed the similarities in patterns of gene expression of GJB2 and several KRTs associated with cell proliferation, it might indicate co-expression partners. And the upregulation of GJB2 gene in this study was corresponding to the abnormal characteristics of the skin similar to those in human and mouse (De Benedetto et al., 2012; Djalilian et al., 2006; Goliger and Paul, 1995; Hivnor et al., 2004). This congruence supports the use of dog as models to study human AD and other skin diseases that arise from the upregulation of this gene. GJB2 may also be used to determine therapeutic efficacy of drugs treated skin diseases that related to cell proliferation as in human psoriasis (Shaker and Abdel-Halim, 2012). In addition, CAD was reported to be associated with the upregulation of a number of genes in a cornified envelope group, including *IVL* and *FLG* (Theerawatanasirikul et al., 2012[c]). Since TGM1 was the linker of the CE proteins, the upregulation of TGM1gene in lesional skin in the present study was corresponding with the previous gene expression of the CE group. From the hierarchical clustering, TGM1 expression was in the same supercluster with IVL and FLG. Since TGM1 expression was not significantly observed in non-lesional skin, the upregulated gene might reflect the clinical phenotypes which should be further studied. ZONAB was found to be downregulated in the non-lesional skin and it tended to show low expression in lesional skin too. ZONAB has been reported to regulate epithelial cell proliferation in canine kidney and mammary cell lines (Sourisseau et al., 2006). However, we observed the lower expression of ZONAB in non-lesional skin. Hence, the role of ZONAB in CAD is needed to be further investigated.

6. Conclusion and Suggestion for the future work

Taken together, these studies establish an intriguing correlation between increased GJB2 and TGM1 expression and CAD, including the possible associations of GJB2 and cell proliferation, and TGM1 and CE proteins. For the future work, since we observed the upregulation of GJB2 and TGM1 in CAD, the clinical application of GJB2 and TGM1 expression as potential diagnostic and/or therapeutic markers for CAD should be investigated.

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8. Research Output

The manuscript is accepted to be published in the Thai Journal of Veterinary Medicine as an original article, Vol 44(3), September 2014. (Article in Press)

9. Appendix

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Association of gap junction beta 2 and transglutaminase 1 gene expression with canine atopic dermatitis

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Abstract

Atopic dermatitis is a common pruritic inflammatory skin disease in humans and dogs. Cell junction and cornified envelope are groups of proteins that are crucial for the formation and stability of the skin barrier. The purpose of this study was to investigate gene expression in cell junction and cornified envelope groups in canine atopic dermatitis (CAD) in small breed dogs. Skin biopsy was performed from 10 lesional CAD, 9 non-lesional CAD cases and 11 normal dogs and subjected to quantitative reverse transcription-polymerase chain reaction. Several cell junction genes were evaluated, including claudin-1, occludin, zonula occludens-1 and -2, zonula occludens-1-associated nucleic acid binding protein, cingulin, gap junction beta 2 and e-cadherin together with transglutaminase 1, a cross-linker of the cornified envelope. An upregulation of gap junction beta 2 and transglutaminase 1 was significantly observed in lesional skin. In conclusion, the present study demonstrates the expression of gap junction beta 2 and transglutaminase 1 in CAD. This is the first report of an association of cell junction and transglutaminase 1 genes with CAD.

Keywords: canine atopic dermatitis, cell junction, dog, gap junction beta 2, gene expression, transglutaminase 1

ความสัมพันธ์ของการแสดงออกของจีนแก็ปจังชันเบต้า-2 และ ทรานส์กลูตามิเนส-1 กับโรคผื่นภูมิแพ้ผิวหนังในสุนัข

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บทคัดย่อ

โรคผื่นภูมิแพ้ผิวหนังเป็นโรคที่ทำให้ผิวหนังอักเสบและคันที่พบได้บ่อยทั้งในคนและสุนัข โปรตีนกลุ่มรอยต่อระหว่างเซลล์และกลุ่มคอร์นิไฟด์เอนเวลโลปมีความสำคัญในการสร้างและความอยู่ตัวของผิวหนัง วัตถุประสงค์ของการศึกษาครั้งนี้คือต้องการตรวจสอบการแสดงออกของจีนกลุ่มรอยต่อระหว่างเซลล์และจีนที่เกี่ยวข้องกับการสร้างคอร์นิไฟด์เอนเวลโลปในโรคผื่นภูมิแพ้ผิวหนังในสุนัขพันธุ์เล็ก โดยทำการตัดชิ้นเนื้อจากผิวหนังสุนัขที่มีรอยโรค 10 ตัว ไม่มีรอยโรค 9 ตัว เปรียบเทียบกับผิวหนังสุนัขปกติ 11 ตัว นำมาทำปฏิกิริยาลูกโซ่พอลิเมอเรสเรียลไทม์แบบย้อนกลับ จีนกลุ่มรอยต่อระหว่างเซลล์ที่ได้ทำการศึกษาได้แก่ คลาวดิน-1 อ็อกคลูดิน โซนาอ็อกคลูเดนส์-1 และ -2 โซนาอ็อกคลูเดนส์-1 แอสโซสิเอดเต็ดนิวคลีอิกแอซิดบายดิงโปรตีน ซินกูลิน แก็ปจังชันเบต้า-2 และอีแคทฮีริน และได้ทำการศึกษาจีนทรานส์กลูตามิเนส-1 ซึ่งมีหน้าที่เชื่อมโปรตีนในกลุ่มคอร์นิไฟด์เอนเวลโลป จากการศึกษาพบการแสดงออกของจีนแก็ปจังชันเบต้า-2 และ ทรานส์กลูตามิเนส-1 เพิ่มขึ้นอย่างมีนัยสำคัญ ณ ผิวหนังที่มีรอยโรค สรุปได้ว่าการศึกษาครั้งนี้ได้รายงานการแสดงออกของจีนแก็ปจังชันเบต้า-2 ทรานส์กลูตามิเนส-1 ในโรคผื่นภูมิแพ้ผิวหนังในสุนัข และได้แสดงความสัมพันธ์ระหว่างรอยต่อระหว่างเซลล์และทรานส์กลูตามิเนส-1 กับโรคผื่นภูมิแพ้ผิวหนังในสุนัขเป็นครั้งแรก

คำสำคัญ: โรคผื่นภูมิแพ้ผิวหนังในสุนัข แก็ปจังชั่นเบต้า 2 ทรานส์กลูตามิเนส 1 รอยต่อระหว่างเซลล์ การแสดงออกของจีน สุนัข

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Introduction

Atopic dermatitis (AD) or atopic eczema is the common multifactorial skin disease recognized in dogs (Hillier and Griffin, 2001) and human (Wadonda-Kabondo et al. 2003). Canine atopic dermatitis (CAD) is originally defined as "*genetically predisposed inflammatory* and *pruritic* allergic *skin disease* with *characteristic clinical features associated* with *IgE antibodies most commonly directed against environmental allergens"* (Halliwell, 2006). *For the canine* atopic-like dermatitis, it is the CAD without IgE prerequisite. Both food-induced and non-food induced atopic dermatitis or canine atopic dermatitis senso strictu has been mentioned. (Favrot et al., 2010; Olivry, 2010).

CAD is associated with the defect of skin barrier which prevents allergens from penetrating the skin. A cell junction or intercellular bridge is a contact of adjacent cells or between a cell and the extracellular matrix especially in epithelial tissues, contributing the paracellular barrier and helping the paracellular transport. In vertebrate, cell junctions can be functionally classified into 4 types, including i.) anchoring junctions ii.) gap or communicating or channel-forming junctions, iii.) tight or occluding junctions, and iv.) signal-relaying junctions (Alberts et al., 2007). Anchoring-type junctions function to hold cells and provide strong structural cohesion between adjacent cells in tissues that have to stand constant mechanical stress e.g. skin and cardiac muscle in mouse and/or human (Kuwahara et al., 2001; Mezzano and Sheikh, 2012). Protein members in this group include cadherins (CDH), catenins, integrins, etc. Gap junctions are intercellular channels that allow communication of small molecules (metabolites, second messengers, and ions, up to a molecular weight of about 1000 daltons between cells (Sosinsky and Perkins, 2000), leading to cell proliferation, cell death, tumor suppression, and action potential in several organs such as rat heart, mouse brain, chicken retina, and mouse skin (Becker et al., 2002; Clarke et al., 2006; Kretz et al., 2004; Teubner et al., 2001). There are several gap junction proteins such as gap junction alpha 1 protein (GJA1) or connexin 43 (Cx43) and gap junction beta 2 protein (GJB2) or Cx26 (Bruzzone, 2001). Tight junctions (TJs) are intercellular junction that adhere two neighboring cells near the apical side of cells. A number of TJ proteins have been identified in mammalian epidermis including occludin (OCL), cingulin (CGN), zonula occludens-proteins (ZOs), claudins (CLDNs), and junctional adhesion molecules (JAMs) (Kirschner et al., 2010). CGN can form complexes with ZO-1, ZO-2 and JAM-1 (Guillemot and Citi, 2006). In skin diseases which are characterized by impaired skin barrier function, altered proliferation/differentiation of the epidermis and/or infiltration of inflammatory cells, alterations of the expression patterns of TJ genes were described such as

the downregulation of CLDN1, CLDN3, and JAM-1 and upregulation of ZO-1 and OCLN in human psoriasis (Kirschner et al., 2009; Watson et al., 2007). Upregulation of ZO-1 and OCLN proteins in early stage of *Staphylococcus aureus* infection and upregulation of CLDN-1 and OCLN proteins in mouse skin exposed to UV radiation were also shown (Ohnemus et al., 2008*).* In atopic dermatitis, CLDN1 and CLDN23 expression were decreased in HAD patients whereas GJB2 was inversely upregulated (De Benedetto et al., 2012). The association of TJ gene expression with CAD has not yet been demonstrated. The signal-relaying junctions such as chemical synapses and immunological synapses play an important role in relaying signals from cell to cell and three groups mentioned previously also have signal functions (Alberts et al., 2007*)*.

The cornified envelope (CE) is a protein complex layer beneath the plasma membrane of mammalian epidermis in terminal differentiation of the skin. It helps protect skin cells against water loss and infection and maintain epidermal structure integrity (Hohl, 1990; Reichert et al., 1993). Gene expression of a number of CE proteins, including involucrin (IVL), filaggrin (FLG) together with other epidermal differentiation and proliferation markers such as keratin 5 (KRT5) and KRT 10 have been reported in normal dog skin in different breeds and coat types (Theerawatanasirikul et al., 2012[a]) and in CAD (Theerawatanasirikul et al., 2012[b]; Theerawatanasirikul et al., 2012[c]). Transglutaminase 1 (TGM1) is an enzyme that facilitates cross-linking of the CE proteins in mature keratinocytes by catalyzing formation of ε-(γ-glutamyl)-lysine cross-links in proteins (Greenberg et al., 1991). However, the association of TGM1 and CAD has not yet been demonstrated. The objective of this study, therefore, was to characterize the gene expression patterns of *CLDN1, CGN, OCL, CDH1, GJB2* and *TGM1* genes in lesional atopic, non-lesional atopic and healthy canine skin by the quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The study would give better understanding of the CAD disease.

Materials and methods

Animals

Nineteen atopic dogs from private small animal clinics, fulfilled at least 5 signs of the diagnostic criteria for CAD (Favrot et al., 2010; Olivry 2010), including onset of signs under 3 years of age, dog living mostly indoors, glucocorticoid-responsive pruritus, pruritus sine materia at onset, affected front feet and/or ear pinnae, and nonaffected ear margins and/or dorso-lumbar area. This group comprised of eleven Poodles (1 male and 10 females), six Shih tzus (5 males and 1 female)

and two Pugs (1 male and 1 female), with a mean age of seven years (age range 2–11 years). Eleven healthy control dogs comprised of seven Poodles (1 male and 6 females), three Shih tzus (1 male and 2 females) and one male Pug, with a mean age of seven years (age range 1–10 years).

Skin biopsies and tissue samples

Skin specimens were all taken from the ventral area of each dog to minimize variations due to body location. Lesional samples of erythematous, macular-papular dermatitis and lichenification were selected from the affected areas. Non-lesional samples were obtained from the clinically unaffected skin of atopic dogs whereas control samples were from clinically normal dogs. Punch skin biopsies (6 mm) were obtained after local anesthesia with 2% lidocaine and, then, sutured routinely. Subcutaneous fat was stripped off. The biopsy was immersed in RNALater solution (Life Technologies, Carlsbad, CA) overnight at 4^{o}C and stored at -20^{o}C until being processed for quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). The sample collection and processing procedures were approved by the Chulalongkorn University Animal Care and Use Committee (CU-ACUC), Thailand.

RNA Extraction

The skin tissues in RNALater solution were disrupted in liquid nitrogen to maintain a low temperature. Total RNA was extracted from the skin tissues by homogenization with Trizol reagent (Life Technologies, Carlsbad, CA) and phenol/chloroform/isopropyl alcohol. Subsequently, genomic DNA traces were removed from the RNA with Turbo DNase (Ambion, Austin, TX) to purify the total RNA according to the instructions. The DNase-treated RNA quality and concentration were analyzed using a NanoDrop ND-1000 Spectrophotometer V3.7 (Thermo Fisher Scientific, Waltham, MA).

Quantitative reverse transcriptase PCR

The SuperScript III First-strand synthesis system for RT-PCR (Life Technologies, Carlsbad, CA) was used to synthesize cDNA according to the manufacturer's instructions. Briefly, one microgram of RNA was reverse transcribed in a 20 µL reaction containing 50 ng random primers, 40U RNase inhibitor and 200U Superscript III enzyme. The Rotor Gene 3000 Thermal Cycler (Qiagen, Hilden, Germany) was used to perform quantitative PCR. Except for the housekeeping gene (HKG), *RPS19*, which has been previously described as a suitable HKG for CAD (Schlotter et al., 2009; Theerawatanasirikul et al., 2012^{c}), all primers were designed by the Primer 3 program version 0.4.0 (http://frodo.wi.mit.edu/). Primer pairs were sequenced for specificity and uniqueness

in the dog genome (CanFam2.0, May 2005 assembly). The primers sequences, melting temperatures and amplicons are depicted in Table 1. PCR reactions were performed in a 10 μL volume containing 1x KAPA SYBR Fast qPCR Master Mix Universal (KAPA Biosystems, Cambridge, MA), 200 nM of each primer and the cDNA template. Thermal cycling conditions were as follows: 95 ^{o}C for 2 min for one cycle followed by 40 cycles at 95 ^{o}C for 3 s, 60 ^{o}C for 20 s and 72 ^{o}C for 1 s. Each reaction was performed in duplicate in 3 independent runs. Data from the FAM/SYBR channel operating at an excitation maximum 495 nm and an emission maximum 520 nm was evaluated. A melting curve analysis was used to determine the purity of the amplified products. Relative expression levels were analyzed by the REST-384 (Relative Expression Software Tool) software. Standard curves were generated for each assay as previously demonstrated and threshold cycles of all targets in test samples were normalized to the corresponding *RPS19* levels in control samples (Pfaffl, 2001; Pfaffl et al., 2002; Theerawatanasirikul et al., 2012[c]).

Statistic analysis

The data was analyzed in REST 384 software, using a pair wise fixed reallocation randomization test to test for significance between groups. Results with a p value <0.05 were considered significant. Hierarchical clustering was performed to analyze the pattern of similarity in gene expression in lesional skin in order to identify gene clusters, using Multiexperiment Viewer (MeV) program, version 4.8 (Saeed et al., 2003; Saeed et al., 2006).

Results

For qRT-PCR experiment, *GJB2 and TGM1* expressed up-regulation in lesional skin compared to normal skin with fold changes 8.081 (p = 0.007) and 3.859 (p = 0.038), respectively). *GJB2* also showed up-regulation in lesional skin compared to non- lesional skin with fold change 5.757 (p = 0.025). Lower expression of *ZONAB* was observed in non-lesional skin compared to normal skin (Table 2) with fold change 0.379 (p = 0.003). For hierarchical clustering in lesional skin, when compared with gene expression patterns of *FLG*, *IVL*, other *KRT*s in a previous report (Theerawatanasirikul et al., 2012[c]), *GJB2*, *TGM1*, and *KRT14* were clustered together in one sub-cluster and *KRT1*, *KRT5* and *KRT17* in the other. However both were placed in the same super-cluster with *IVL* and *FLG* whereas *KRT10* and *KRT2*e were clearly different (Fig 1).

Discussion

In the present study, we demonstrate the gene upregulation of *GJB2* and *TGM1* in CAD and this is the first report of an association of cell junction and transglutaminase 1 genes with CAD. Gap junction-mediated cell communication plays a role in maintaining a uniform epidermal thickness with the balance of cell proliferation and differentiation. Several reports showed the association of an increase in the number of keratinocytes and/or the thickening of the epidermis with the induction of GJB2 expression either at the mRNA or protein levels, including a lesion of human skin treated with retinoic acid (Masgrau-Peya et al., 1997), human psoriatic lesions (Hivnor et al., 2004; Labarthe et al., 1998; Lucke et al., 1999; Shaker and Abdel-Halim, 2012), human porokeratotic lesions (Hivnor et al., 2004), tape-stripped epidermis and viral warts (Lucke et al., 1999). In mouse epidermis, GJB2 expression rapidly increased in hyperproliferative wound epidermis (Djalilian et al., 2006; Goliger and Paul, 1995). In mouse skin papillomas, expression of both GJB2 and GJA1 was elevated in the proliferating neoplasms (Sawey et al., 1996). GJB2 was also upregulated in HAD (De Benedetto et al., 2012). In CAD, the hyperproliferation and aberrant keratinocyte differentiation resulted in the thickening of the epidermis (Theerawatanasirikul et al., 2012[b]) Since the hierarchical clustering showed the similarities in patterns of gene expression of *GJB2* and several *KRT*s associated with cell proliferation, it might indicate co-expression partners. And the upregulation of *GJB2* gene in this study was corresponding to the abnormal characteristics of the skin similar to those in human and mouse (De Benedetto et al., 2012; Djalilian et al., 2006; Goliger and Paul, 1995; Hivnor et al., 2004). This congruence supports the use of dog as models to study human AD and other skin diseases that arise from the upregulation of this gene. GJB2 may also be used to determine therapeutic efficacy of drugs treated skin diseases that related to cell proliferation as in human psoriasis (Shaker and Abdel-Halim, 2012). In addition, CAD was reported to be associated with the upregulation of a number of genes in a cornified envelope group, including *IVL* and *FLG* (Theerawatanasirikul et al., 2012[c]). Since TGM1 was the linker of the CE proteins, the upregulation of *TGM1* gene in lesional skin in the present study was corresponding with the previous gene expression of the CE group. From the hierarchical clustering, *TGM1* expression was in the same supercluster with *IVL* and *FLG*. Since *TGM1* expression was not significantly observed in non-lesional skin, the upregulated gene might reflect the clinical phenotypes which should be further studied. *ZONAB* was found to be downregulated in the non-lesional skin and it tended to show low expression in lesional skin too. ZONAB has been reported

to regulate epithelial cell proliferation in canine kidney and mammary cell lines (Sourisseau et al., 2006). However, we observed the lower expression of ZONAB in non-lesional skin. Hence, the role of ZONAB in CAD is needed to be further investigated. Taken together, these studies establish an intriguing correlation between increased *GJB2* and *TGM1* expression and CAD, including the possible associations of *GJB2* and cell proliferation, and *TGM1* and CE proteins. For the future work, since we observed the upregulation of GJB2 and TGM1 in CAD, the clinical application of GJB2 and TGM1 expression as potential diagnostic and/or therapeutic markers for CAD should be investigated.

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Table 1 Primers used in the present study. Indicated are sequences, annealing temperatures in real-time PCR reactions and expected product sizes.

Genes	Primers (5'to 3')	Amplicon (bp)	Accession number
CGN	Fwd 5'-agctcggatgaggagtttga-3' Rev 5'-agaggcaagcctgtctacca-3'	277	DQ910799
CLDN1	Fwd 5'-ggccactattggcatgaagt-3' Rev 5'-atgttgtttttcggggacag-3'	284	XM845155.2
CLDN23	Fwd 5'-gtggacgtggagctgtacc-3' Rev 5'-cggtgggtgtaccaggac-3'	293	XM003639565.1
CDH1	Fwd 5'-ggtgctcacatttcccagtt -3' Rev 5'-aaatgggcctttctcgtttt-3'	100	NM001197148.1
GJB2	Fwd 5'-aaatatgggccggatagacc-3' Rev 5'-tcccaagcaagctccttaaa-3'	180	NM001197148.1
OCLN	Fwd 5'-catggtgattgtggcttttg-3' Rev 5'-ggaggaggcatgtcttgtgt -3'	180	NM001003195.1
ZO-1	Fwd 5'-cggtaccagctcctctcttg-3' Rev 5'-cggtttggtggtctgaaagt-3'	153	NM001003140.1
ZO-2	Fwd 5'-caattcagcatcagcaagga-3' Rev 5'-gctcatccagctcattgtca-3'	198	NM001003204.1
ZONAB	Fwd 5'-cggttcatcgaaatccaact-3' Rev 5'-atggaacttcaggtgccttg-3'	234	AF171061.1
TGM1	Fwd 5'-gcaagaaggaagtggtgctc -3' Rev 5'-acggaaggtatgctgtttgg-3'	167	AF262219.1
RPS19	Fwd 5'-ccttcctcaaaaa/gtctggg-3' Rev 5'-gttctcatcgtagggagcaag-3'	95	XM533657

Table 2 Fold change in gene-specific mRNA expression in CAD lesional, CAD non-lesional and control dog skin compared to *RPS19* expression. *GJB2* and *TGM1* express up-regulation in lesional skin compared to normal skin. *GJB2* also shows up-regulation in lesional skin compared to non- lesional skin. Lower expression of *ZONAB* is observed in non-lesional skin compared to normal skin.

Gene	lesion vs. normal		non-lesion vs. normal		lesion vs. non-lesion	
	Fold change	p value*	Fold change	p value*	Fold change	p value*
CGN	0.711	0.653	1.927	0.314	0.431	0.261
CLDN1	1.330	0.664	2.077	0.178	0.640	0.534
CLDN23	0.665	0.693	0.704	0.632	0.946	0.954
CDH1	1.739	0.363	0.889	0.756	1.955	0.261
GJB2	8.081**	0.007	1.404	0.531	5.757**	0.025
OCLN	0.895	0.883	1.278	0.537	0.700	0.652
ZO-1	8.397	0.499	88.364	0.219	0.095	0.440
ZO-2	1.747	0.367	1.745	0.175	1.001	0.998
ZONAB	0.503	0.333	0.379**	0.003	1.327	0.709
TGM1	3.859**	0.038	2.342	0.073	1.648	0.439

*Pair wise fixed reallocation randomization test normalized by reference gene

**Significantly different at $p < 0.05$

Figure 1 Hierarchical clustering of genes in lesional skin. d = dog.