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Association of Gap Junction Beta 2 and Transglutaminase 1 Gene Expression with Canine Atopic Dermatitis

Gunnaporn Suriyaphol1* Sirin Theerawatnasirikul2 Piyarat Chansiripornchai3

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 dog breeds. 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 the 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 on the 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

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Introduction

Atopic dermatitis (AD) or atopic eczema is a 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 have 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 (CDH1), 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 (GJα1) or connexin 43 (Cx43) and gap junction beta 2 protein (GJB2) or Cx26 (Bruzzone, 2001). Tight junctions (TJs) are intercellular junctions 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 (ZO1, ZO2, ZO3), claudins (CLDNs), and junctional adhesion molecules (JAMs) (Kirschner et al., 2010). CGN can form complexes with ZO-1, ZO-2 and JAM-1 (Guillen 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 human atopic dermatitis (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 the 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) and in CAD (Theerawatanasirikul et al., 2012; Theerawatanasirikul et al., 2012). 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 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, fulfilling 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, living mostly indoors, glucocorticoid-responsive pruritus, pruritus sine materia at onset, affected foot and/or ear pinnae, and non-affected ear margins and/or dorso-lumbar area, were included in the study. This group comprised eleven Poodles (1 male and 10 females), six Shih tzu (5 males and 1 female) and two Pugs (1 male and 1 female), with a mean age of seven years (age ranging from 2–11 years). For the control group, there were eleven healthy dogs comprising seven Poodles (1 male and 6 females), three Shih tzu (1 male and 2 females) and one male Pug, with a mean age of seven years (age ranging from 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 affected areas. Non-lesional samples were obtained from 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 RNA Later solution (Life Technologies, Carlsbad, CA) overnight at 4°C and stored at -20°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:** 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. 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), 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°C for 2 min for one cycle followed by 40 cycles at 95°C for 3 s, 60°C for 20 s and 72°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 of 495 nm and an emission maximum of 520 nm was evaluated. A melting curve analysis was used to determine the purity of the amplified products. Relative expression levels were analyzed by REST-384 (Relative Expression Software Tool) software. Standard curves were generated for each assay as previously demonstrated and threshold cycles of all targets in the test samples were normalized to the corresponding RPS19 levels in the control samples (Pfaffl, 2001; Pfaffl et al., 2002; Theerawatanasirikul et al., 2012).

**Statistical 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).

### Table 1

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers (5’to 3’)</th>
<th>Amplicon (bp)</th>
<th>Accession number</th>
</tr>
</thead>
</table>
| CGN   | Fwd 5’-agtctgagaggaatg-3’  
      | Rev 5’-tgtagggaaagctct-3’  | 277  
      | DQ910799    |
| CLDN1 | Fwd 5’-gccataattgcatga-3’  
      | Rev 5’-gtgttggcggcgtt-3’  | 284  
      | XM845155.2  |
| CLDN23| Fwd 5’-gttgagaggcctg-3’    
      | Rev 5’-cggtggtgcatgcc-3’  | 293  
      | XM00369565.1|
| CDS1  | Fwd 5’-gctgttaacttctg-3’   
      | Rev 5’-aatggggctttctg-3’  | 100  
      | NM001197148.1|
| GJB2  | Fwd 5’-aatatgaggccagtac-3’ 
      | Rev 5’-tcctcaagacttctaa-3’| 180  
      | NM001197148.1|
| OCLN  | Fwd 5’-cggtggacagtcgct-3’  
      | Rev 5’-agggagacgctcttg-3’| 180  
      | NM00103195.1|
| ZO-1  | Fwd 5’-cggccagccctttgc-3’  
      | Rev 5’-cggtggtgcatgcc-3’  | 153  
      | NM001003140.1|
| ZO-2  | Fwd 5’-cggctgacaggtcag-3’  
      | Rev 5’-gcctcagcatttca-3’  | 198  
      | NM001003204.1|
| ZONAB | Fwd 5’-cggctatcaaatccaa-3’ 
      | Rev 5’-agcagactgctgctt-3’ | 234  
      | AF171061.1  |
| TGM1  | Fwd 5’-gaagagggagggtttg-3’ 
      | Rev 5’-cgggggctgtttg-3’   | 167  
      | AF262219.1  |
| RPS19 | Fwd 5’-ctctctctctaa/gtcggg-3’  
      | Rev 5’-gtttctctgagggaggag-3’| 95   
      | XM533657    |
Results

For the qRT-PCR experiment, GJB2 and TGM1 expressed upregulation in the lesional skin compared to the normal skin with fold changes of 8.081 \((p = 0.007)\) and 3.859 \((p = 0.038)\), respectively. GJB2 also showed upregulation in the lesional skin compared to the non-lesional skin with a fold change of 5.757 \((p = 0.025)\). Lower expression of ZONAB was observed in the non-lesional skin compared to the normal skin (Table 2) with a fold change of 0.379 \((p = 0.003)\). For the hierarchical clustering in the lesional skin, when compared with gene expression patterns of FLG, IVL, other KRTs in a previous report (Theerawatanasirikul et al., 2012c), 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).

Discussion

In the present study, we demonstrate the gene upregulation of GJB2 and TGM1 in CAD and this is the first report on the 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

Table 2

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 upregulation in lesional skin compared to normal skin. GJB2 also shows upregulation in lesional skin compared to non-lesional skin. Lower expression of ZONAB is observed in non-lesional skin compared to normal skin.

<table>
<thead>
<tr>
<th>Gene</th>
<th>lesional vs. normal</th>
<th>non-lesional vs. normal</th>
<th>lesional vs. non-lesional</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fold change</td>
<td>(p) value*</td>
<td>Fold change</td>
</tr>
<tr>
<td>CGN</td>
<td>0.711</td>
<td>0.653</td>
<td>1.927</td>
</tr>
<tr>
<td>CLDN1</td>
<td>1.330</td>
<td>0.664</td>
<td>2.077</td>
</tr>
<tr>
<td>CLDN23</td>
<td>0.665</td>
<td>0.693</td>
<td>0.704</td>
</tr>
<tr>
<td>CDH1</td>
<td>1.739</td>
<td>0.363</td>
<td>0.889</td>
</tr>
<tr>
<td>GJB2</td>
<td>8.081**</td>
<td>0.007</td>
<td>1.404</td>
</tr>
<tr>
<td>OCLN</td>
<td>0.895</td>
<td>0.883</td>
<td>1.278</td>
</tr>
<tr>
<td>ZO-1</td>
<td>8.397</td>
<td>0.499</td>
<td>88.364</td>
</tr>
<tr>
<td>ZO-2</td>
<td>1.747</td>
<td>0.367</td>
<td>1.745</td>
</tr>
<tr>
<td>ZONAB</td>
<td>0.503</td>
<td>0.333</td>
<td>0.379**</td>
</tr>
<tr>
<td>TGM1</td>
<td>3.859**</td>
<td>0.038</td>
<td>2.342</td>
</tr>
</tbody>
</table>

* 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
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). 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. Moreover, the upregulation of GJB2 gene in this study corresponded 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 treating skin diseases 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). Since TGM1 was the linker of the CE proteins, the upregulation of TGM1 gene in lesional skin in the present study corresponded with the previous gene expression of the CE group. From the hierarchical clustering, TGM1 expression was in the same superclass with IVL and FLG. Since TGM1 expression was not significantly observed in the 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 also tended to show low expression in lesional skin. 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 the 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 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.

Acknowledgements

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References


บทความ

ความสัมพันธ์ของการแสดงออกของจีนแก็ปจังชันเบต้า 2 และ ทรานส์กลูตำมิเนส 1 กับโรคภูมิแพ้ผิวหนังในสุนัข

กรรณาภรณ์ สุริยผล’, ศิรินทร์ ธีระวัฒนศิริกุล, ปิยะรัตน์ จันทร์ศิริพรชัย

โรคภูมิแพ้ผิวหนังเป็นโรคที่ทำให้ผิวหนังอักเสบและคันที่พบได้บ่อยทั้งในคนและสุนัข โปรตีนกลุ่มรอยต่อระหว่างเซลล์และกลุ่มคอร์นิไฟด์มีความสำคัญในการสร้างผิวหนังและความอยู่ตัวของผิวหนัง วัตถุประสงค์ของการศึกษาครั้งนี้คือต้องการตรวจสอบการแสดงออกของจีนกลุ่มรอยต่อระหว่างเซลล์และจีนที่เกี่ยวข้องกับการสร้างคอร์นิไฟด์เอ็นเวลโลปในโรคภูมิแพ้ผิวหนังในสุนัขพันธุ์เล็ก โดยทำการตัดชิ้นเนื้อจากผิวหนังสุนัขที่มีโรคร่อง 10 ตัว ไม่มีโรคร่อง 9 ตัว แบ่งเป็นกลุ่มที่ผิวหนังสุนัขปกติ 11 ตัว และนำชิ้นเนื้อที่ทำการวิเคราะห์ถูกเตรียมไว้ด้วยโปรตีนลูกโซ่定点實體逆時序 Real Time PCR จีนกลุ่มรอยต่อระหว่างเซลล์ที่ศึกษาได้แก่ คลายหน้า-1 อ็อกคลูเดน โซนอ็อกคลูเดน-1 และ -2 โซนอ็อกคลูเดน-1 และโซนโปรตีนที่เกี่ยวกับการสื่อสารช่องปุเตอร์ ซินกูลิน แก็ปจังชันเบต้า 2 และอีแคทฮีริน และได้ทำการศึกษาจำนวนสุนัขพันธุ์เล็ก 1 ชนิดที่มีหน้าที่มีโรคร่องในกลุ่มคอร์นิไฟด์เอ็นเวลโลป จากการศึกษาการแสดงออกของจีนแก็ปจังชันเบต้า 2 และ ทรานส์กลูตำมิเนส 1 เพิ่มขึ้นอย่างมีนัยสำคัญ ณ ระดับที่มีความสัมพันธ์ สรุปได้ว่าการศึกษาครั้งนี้ได้รายงานการแสดงออกของจีนแก็ปจังชันเบต้า 2 และ ทรานส์กลูตำมิเนส 1 ในโรคภูมิแพ้ผิวหนังในสุนัข และได้แสดงความสัมพันธ์ระหว่างรอยต่อระหว่างเซลล์และกับร่องอักเสบของผิวหนังเป็นครั้งแรก

คำสำคัญ: โรคภูมิแพ้ผิวหนังในสุนัข รอยต่อระหว่างเซลล์ สุนัข แก็ปจังชันเบต้า 2 การแสดงออกของจีน ทรานส์กลูตำมิเนส 1

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