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Alterations of keratins, involucrin and filaggrin gene expression in canine atopic dermatitis

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ABSTRACT

Canine atopic dermatitis (CAD) is a common allergic skin disease in dogs, associated with a defective epidermal barrier. In this study we investigated the alterations in skin keratinocyte proliferation and differentiation in CAD by quantitative reverse transcription-polymerase chain reaction. Gene expression of keratin (*KRT*) markers of proliferative and differentiated keratinocytes, together with that of cornified envelope proteins, involucrin (*IVL*) and filaggrin (*FLG*), were evaluated. An upregulation of *KRT5* and *KRT17* in both lesional and non-lesional AD skin was observed ($p < 0.05$) whereas *KRT2e, KRT14, IVL* and *FLG* expression were significantly increased only in lesional AD skin ($p < 0.05$). Additionally, the expression levels of *KRT5, KRT14, KRT17* and *IVL* in CAD were strongly correlated. In conclusion, the expression of the majority of the studied keratins, as well as *IVL* and *FLG* is increased in CAD with close correlation between the proliferative keratins. This is the first report of a correlation of *KRT* and *IVL* genes with CAD.

1. Introduction

Atopic dermatitis (AD) is a common allergic skin disease recognized in dogs and humans (Rothe and Grant-Kels, 1996; Hillier and Griffin, 2001). Canine atopic dermatitis (CAD) is a 'genetically predisposed inflammatory and pruritic allergic skin disease with characteristic clinical features associated with IgE antibodies most commonly directed against environmental allergens' (Halliwell, 2006). Approximately 10% of the canine population is affected by CAD (Scott et al., 2001). Disturbances in epidermal differentiation and proliferation lead to a defect in skin barrier permeability that enhances the penetration of environmental allergens (Hudson, 2006; Proksch et al., 2009). In CAD, the structural changes of lesional AD skin have been demonstrated to be epidermal hyperplasia, hyperkeratosis, hypergranulosis and spongiosis (Scott, 1981; Olivry et al., 1997). The correlation of the disease with keratin markers has not yet been studied.

Normal epidermal cell proliferation is restricted to the basal layer where several keratin marker proteins such as *KRT5* and *KRT14* are expressed. Basal cells undergo differentiation and maturation and move then into the suprabasal compartments where differentiated keratin markers such as *KRT1* and *KRT10* are expressed, whereas *KRT2e* represents late epidermal differentiation (Leigh et al., 1993; Bloor et al., 2003). In humans, keratins *KRT6*, *KRT16*, and *KRT17* have been associated with epidermal hyperproliferation in various diseases, such as psoriasis, basaloid skin tumors, and during wound healing (Leigh et al., 1995; Machesney et al., 1998; Depianto et al., 2010). As keratinocytes differentiate further, several cornified envelope (CE) proteins are synthesized in the granular layer. These proteins include filaggrin (*FLG*), loricrin, involucrin (*IVL*), and S100 proteins. CE proteins help form the outer most layer of the epidermis, the cornified layer, in the final epidermal differentiation and hydrate the skin. The proliferation and differentiation of keratinocytes are important for CE associated protein formation (Steinert and Marekov, 1997; Candi et al., 2005; Proksch et al., 2008). Humans with AD have decreased expression of *FLG* and *IVL* in skin (Seguchi et al., 1996; Jensen et al., 2004). Although the protein expression of *FLG* and *IVL* in CAD has been studied, the association to CAD is still unclear (Marsella et al., 2009; Chervet et al., 2010).

A problem when studying CAD at the protein level is that available antibodies in commerce tested for specificity in dogs are limited. The anti-human filaggrin antibody demonstrated cross-reactivity with canine epidermal proteins (Marsella et al., 2009). The sequencing of the canine genome (Lindblad-Toh et al., 2005) has enabled the study of canine diseases at the transcript level,

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which is more specific to the proteins of interest. The genes encoding human epidermal differentiation complex have been shown to be generally regulated at the transcription level (Segre, 2006). However, genes involved in CAD have been less intensively studied. The aim of this study was to quantify and investigate the correlation between the expression of *KRT1, KRT2e, KRT5, KRT10, KRT14, KRT17, FLG* and *IVL* at the transcript level in lesional atopic, non-lesional atopic and healthy canine skin. Determining the expression pattern of these genes will help, at least in part, our understanding of this disease.

2. Materials and methods

2.1. Animals

Thirty-one dogs were recruited from private small animal clinics. Twenty dogs with AD, comprising twelve Poodles, six Shih tzus and two Pugs, with a mean age of seven years (age range 2–11 years), and eleven healthy control dogs, comprising seven Poodles, three Shih tzus and one Pug, with a mean age of seven years (age range 1–10 years) were used for this study. The diagnosis of CAD was based on compatible history, clinical signs, the presence of 5 signs or more under Favrot's 2010 criteria, and the exclusion of other causes of pruritus (Favrot et al., 2010; Olivry, 2010). Bacterial and yeast infections and ectoparasite infestations were controlled prior to inclusion. No anti-inflammatory medication was given for at least three weeks prior to examination. Control skin samples were obtained from healthy dogs with no history or clinical signs of skin disease. All samples were obtained with the consent of owners following the ethical guidelines required under the Chulalongkorn University Animal Care and Use Committee (CU-ACUC), Thailand.

2.2. Skin biopsies and tissue samples

A 6-mm skin biopsy of 5-mm depth was taken from the ventral abdomen of each dog to minimize variations due to body location. Lesional samples were taken from the skin of 10 atopic dogs with erythema, maculae, papulae or lichenification. Non-lesional samples were obtained from clinically unaffected skin of other 10 atopic dogs. Control samples were obtained from clinically normal dogs. Biopsies were obtained after local anaesthesia with 2% lidocaine and sutured routinely. Subcutaneous fat was removed. The tissues were maintained in RNALater solution overnight at 4 °C and then stored at −20 °C until processed for quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

2.3. 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 a phenol/chloroform/isopropyl alcohol technique was performed. Subsequently, genomic DNA traces were removed from the RNA with Turbo DNase (Ambion, Austin, TX) to purify the total RNA according to the manufacturer's instructions. The DNase-treated RNA quality and concentration were analyzed using a NanoDrop ND-1000 Spectrophotometer V3.7 (Thermo Fisher Scientific, Waltham, MA).

2.4. Quantitative reverse transcription PCR

cDNA synthesis was performed using the SuperScript III First-strand synthesis system for RT-PCR (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Briefly, one microgram of RNA was reverse transcribed in a 20 μL reaction containing 50 ng random primers, 40 U RNase inhibitor and 200 U Superscript III enzyme. qRT-PCR was performed and analyzed on the Rotor Gene 3000 Thermal Cycler (Qiagen, Hilden, Germany). Prior to this study, potentially suitable reference genes were assessed and *RPS19* was demonstrated to be the most stably expressed among different conditions and therefore the most suitable reference gene to normalization. Primer sequences and cycling details for quantitative determination of the housekeeping gene *RPS19* has been previously described (Brinkhof et al., 2006; Wood et al., 2008; Schlotter et al., 2009). Other primers were developed using Primer 3 version 0.4.0 (http://frodo.wi.mit.edu/). Primer pairs were investigated for specificity and uniqueness in the dog genome (CanFam2.0, May 2005 assembly) by in silico analysis using In-Silico PCR at the University of California, Santa Cruz Genome Bioinformatics Site (http://genome.ucsc.edu/). The primer sequences, accession numbers, 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 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 three independent runs. Data from the FAM/SYBR channel operating at

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

Genes	Accession number	Primers (5′–3′)	Amplicon (bp)
Keratin 1 (*KRT1*)	NM_001003392	Fwd 5′ TCTGGAAGGAGAGGAAAGCA 3′ Rev 5′ ATAACTGCCACCACCGTAGC 3′	157
Keratin 2e (*KRT2e*)	NM_001003386	Fwd 5′ AGCTCCATGTCCTCAAGCAT 3′ Rev 5′ GCCACTTCCAGAGCTGAATC 3′	105
Keratin 5 (*KRT5*)	XM_003433511	Fwd 5′ TCAACCAGAGCCTCCTGACT 3′ Rev 5′ CTTGGTGTCCAGGACCTTGT 3′	164
Keratin 10 (*KRT10*)	NM_001013425	Fwd 5′ TTGAGACGCACTGTTCAAGG 3′ Rev 5′ AGCTCGGATCTGTTGCAGTT 3′	168
Keratin 14 (*KRT14*)	XM_548101	Fwd 5′ CTGAAGGAGGAGCTGGCTTA 3′ Rev 5′ TTCAGTTCCTCGGTCTTGCT 3′	218
Keratin 17 (*KRT17*)	XM_548100	Fwd 5′ CAACAGCGAGCTGGTACAGA 3′ Rev 5′ TGTCTCAGCCAGGCTACCTT 3′	130
Involucrin (*IVL*)	M34442	Fwd 5′ AAAGAAGAGCAGGTGCTGGA 3′ Rev 5′ TGCTCACTGGTGTTCTGGAG 3′	203
Filaggrin (*FLG*)	ENSCAFG00000023034	Fwd 5′ GATGACCCAGACACTGCTGA 3′ Rev 5′ TGGTTTTGCTCTGATGCTTG 3′	158
Ribosomal protein S19	XM_533657	Fwd 5′ CCTTCCTCAAAAA/GTCTGGG 3′ Rev 5′ GTTCTCATCGTAGGGAGCAAG 3′	95