proteases matriptase which plays an important role in proteolytically cleavage of profilaggrin to release multiple functional FLG repeat peptide units (List et al 2003; O'Regan et al 2008). LEKTI protein will keep balance of FLG amount. As far as we know, the association of LEKTI and FLG in CAD has not been demonstrated.

The Canine Atopic Dermatitis Extent and Severity Index (CADESI), adapted from the human SCORing Atopic Dermatitis (SCORAD), is recommended by the International Task Force on Canine Atopic Dermatitis for the evaluation of the extent and severity of skin lesions in CAD. The third version of CADESI (CADESI-03) scale consists of the evaluation of 4 different lesions (erythema, excoriations, lichenification, and self induced alopecia) at 62 body sites with a severity scale varied from 0 to 5 as follows: none [0], mild [1], moderate [2,3], and severe [4,5]. Hence, the maximal achievable score was 62 x 4 x 5 = 1240 (Olivry et al 2007).

The aim of this study was to quantify the expression of K5, K10, FLG and IVL in lesional atopic, non-lesional atopic and healthy canine skin either at the protein and gene levels. The keratinocyte proliferation was also studied by Ki-67 expression. The association of the gene and protein expressions in atopic skin with the CADESI-03 score were investigated. Determining the expression pattern of these proteins would help, at least in part, unveil the pathogenesis of CAD.

3. Procedures

Animals

Thirty one dogs were recruited from the private small animal clinics. Table 1 summarized breeds and their health status. The diagnosis of CAD was based on compatible history and clinical signs, exclusion of other causes of pruritus and 5 signs or more under Favrot's 2010 criteria (Favrot et al 2010; Olivry 2010). Bacterial and yeast infections and ectoparasite infestation were controlled prior to inclusion. No anti-inflammatory medication was given for at least 3 weeks prior to examination. Clinical lesions of CAD were scored by using CADESI-03 (Olivry et al 2007). The total score from all clinical signs and body sites was statistically analyzed. Healthy control samples were taken from clinically normal skin and appearances with no history and clinical sign of skin diseases. All animal were obtained under the consents of the dog owners that followed ethical guidelines as required under Chulalongkorn University Animal Care and Use Committee (CU-ACUC), Thailand.

Skin biopsies and tissue samples

A 6-mm skin biopsy specimen of 5-mm depth was taken from the ventral area of each dog to minimize variations due to body location. Lesion samples (n = 10) were selected from the affected areas of erythematous, macular-papular dermatitis and lichenification. Non-lesional samples (n = 10) were taken from clinically unaffected skin of the other atopic dog group whereas control samples (n = 11) were from clinically normal dogs. Biopsy was taken from each dog after local anaesthesia with 2% lidocaine and 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

Table 1 Breeds and health status of dogs included in the study

Breeds	Health Status	Average Age (range, yr)	Numbers
Poodle	CAD	7.8 (3-11)	12
	Healthy control	7.7 (1-10)	7
Shih tzu	CAD	5.8(2-9)	6
	Healthy control	7.3(4-10)	3
Pug	CAD	5.5(5-6)	2
	Healthy control	1	1

tissue processing and paraffin embedding for a routine histopathological and immunohistochemical study. The other half was kept in RNALater solution overnight at 4°C and stored at -20°C until processing for quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

Histology and Immunohistochemistry:

Formalin fixed paraffin embedded (FFPE) sections of 3 microns were placed on glass slides for routine staining with hematoxylin and eosin (HE) or on positively charged slides for IHC. FFPE tissue was deparaffinized with xylene and rehydrated with a series of graded ethanols. After HE staining, the slides were examined microscopically. Ten fields were randomly selected at x200 magnification and the epidermal thickness was

assessed by measurement of total thickness, nucleated epidermis and S. corneum, using Image-Pro® PLUS 6.0 Programming software (Media Cybernetics, Bethesda, MD).

Immunohistochemistry was performed on replicate sections. For Ki-67 and LEKTI antigen retrieval, the slides were incubated in citrate buffer (0.01 M, pH 6.0) at 95°C for 40 min and for the other 4 antigens, the slides were trypsinized by 1.0% trypsin (Merck, Rockland, MA) at 37°C for 15 min. Endogenous peroxidase was quenched by incubating the slides in 3% hydrogen peroxide in dH_2O for 5 min. Nonspecific immunoglobulin binding was blocked with 2% bovine serum albumin at room temperature for 20 min (Merck, Rockland, MA). A mouse monoclonal anti-human Ki-67 antibody (MIB-1) (Dako, Glostrup, Denmark) at dilution 1:200 was used for investigation of the epidermal proliferation. To assess the localization of the CE proteins and epidermal differentiation, a panel of monoclonal and polyclonal antibodies was used as follows: a rabbit polyclonal against human filaggrin, mouse monoclonal against canine involucrin (SY5), mouse monoclonal against canine cytokeratin 10 (DE-K10) antibodies at dilutions of 1:2000, 1:1000 and 1:600, respectively (Abcam, Cambridge, UK), and a mouse monoclonal anti-human cytokeratin 5 clone XM26 antibody (prediluted, Diagnostic Biosystems, Pleasanton, CA) and a rabbit polyclonal anti-human LEKTI antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:3000. All antibodies were incubated at 4°C overnight. A Polymer-based nonavidin–biotin system EnVision (Dako, Glostrup, Denmark) was used for immunolabeling at room temperature for 30 minutes followed by a 3, 3'-diaminobenzidine tetrahydrochloride (DAB) solution (ZYMED Laboratories, San Francisco, CA). Slides were counterstained with Mayer's hematoxylin. A positive control

was a human skin section and a negative control slide was healthy dog skin section prepared without the primary antibody.

Quantitative Image Analysis:

Epidermal proliferation in each section was determined by counting the number of keratinocytes staining positive for Ki-67 on the epidermis of each biopsy. The average numbers of positive cells were calculated for the epidermis in the unit 'Positive cells per linear mm of total epidermal surface length'.

For quantitative assessment of filaggrin, involucrin, keratin10 and keratin 5 immunostaining, the staining results were evaluated by a semi-quantitative manual scoring method and by image analysis software. For semi-quantitative manual scoring, the manual scoring of 5 antibodies of positive areas was done by classification into 4 different levels of intensity: 0 (negative), + (mild), ++ (moderate), +++ (strong). Since a number of cells were not positively stained in some compartments of skin, the proportion of positively stained area of the epidermis to negative cells of epidermis was included in this study. The proportion was estimated into four different levels (marked A–D): low proportion (<25% of positive epidermis, A); moderate proportion (25-50% of positive epidermis, B); high proportion (50-75% of positive epidermis, C) and almost all positive areas (more than 75% positive epidermis, D) (Sukjumlong et al 2005). For the image analysis, each sample was performed on 10 randomly selected fields. By using the Image-Pro PLUS 6.0 software (Media Cybernetics, Bethesda, MD), the cytoplasmic staining intensity was divided into four different levels: 0 (negative), 1 (weak), 2

(moderate), and 3 (strong). Percentage of positive area was evaluated from dividing of positive cytoplasmic staining (μm^2) per total area of epidermis (μm^2).The ratio of positive area in combination with intensity score was calculated as 'staining score'. The staining scores were calculated from the summation of (intensity score x percentage of area stained in each level) divided by 100 and the mean of the total scores was calculated and used for analysis.

RNA Extraction

The skin tissues in RNALater solution were disrupted in liquid nitrogen to maintain a low temperature. Total RNA was extracted from skin tissues by homogenization with Trizol reagent (Life Technologies, Carlsbad, CA) and was performed using a phenol/chloroform/isopropyl alcohol technique. Subsequently, genomic DNA traces were removed from RNA with Turbo DNase (Ambion, Austin, TX) to purify total RNA according to the instructions. 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

cDNA synthesis was performed using 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, 40U RNase inhibitor and 200U Superscript III enzyme. Real-time

PCR was performed and analyzed on the Rotor Gene 3000 Thermal Cycler (Qiagen, Hilden, Germany). Primers were developed using software from 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) in silico using In-Silico PCR at the UCSC Genome Bioinformatics Site (http://genome.ucsc.edu/). The primers sequences, melting temperatures and amplicons were depicted in Table 2. 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 FAM/SYBR channel operating at excitation maximum 495 nm and emission maximum 520 nm was evaluated to ensure that PCR amplification took place. A melting curve analysis was used to determine purity of the amplified products. Prior to this study, potentially suitable reference gene was assessed and RPS19 was demonstrated to be the most stably expressed among different conditions and therefore the most suitable reference genes to normalize the rest of the samples. Primer sequences and cycling details for quantitative determination of the housekeeping gene *RPS19* has been described before (Brinkhof et al 2006; Wood et al 2008). Relative expression levels were calculated by the REST-384 (Relative Expression Software Tool) software with a detection threshold at 0.1 (Pfaffl et al 2002). Standard curves were generated for each assay using the fluorescent data from 10-fold serial dilutions of total RNA of the same normal dog and the PCR efficiency was calculated

Table 2 Primers used for reverse transcriptase quantitative PCR. Indicated are sequences, annealing temperatures in PCR reactions and expected product sizes.

Genes	Primers (5'to 3')	Annealing temp (°C)	Amplicon (bp)
Involucrin (IVL)	Fwd 5'-AAA GAA GAG CAG GTG CTG GA-3'	60	203
	Rev 5'-TGC TCA CTG GTG TTC TGG AG-3'		
Filaggrin (FLG)	Fwd 5'-GAT GAC CCA GAC ACT GCT GA-3'	60	158
	Rev 5'-TGG TTT TGC TCT GAT GCT TG-3'		
Keratin 5 (K5)	Fwd 5'- TCA ACC AGA GCC TCC TGA CT -3'	60	164
	Rev 5'- CTT GGT GTC CAG GAC CTT GT -3'		
Keratin 10 (K10)	Fwd 5'-TTG AGA CGC ACT GTT CAA GG-3'	60	168
	Rev 5'-AGC TCG GAT CTG TTG CAG TT-3'		
SPINK 5	Fwd 5'-TGA ATT GCG ATG ATT TCA GG-3'	60	178
	Rev 5'-TTG GTC TCA CAT TCC CCT TC-3'		
Ribosomal protein S19 (RPS19)	Fwd 5'-CCT TCC TCA AAA A/GTC TGG G-3'	60	95
	Rev 5'-GTT CTC ATC GTA GGG AGC AAG-3'		

from standard curve slope by a modified delta delta threshold cycle (C_T) method (Pfaffl 2001) and expressed as Target Gene normalized to RPS19. Linearity of the obtained