The Thai Journal of Veterinary Medicine

Volume 42
Issue 3 *September, 2012*

Article 6

9-1-2012

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Original Article

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Abstract

The pathological alterations in skin keratinocyte proliferation and differentiation in canine atopic dermatitis (CAD) were investigated in 20 small breed dogs with CAD (10 of which displayed lesional CAD skin and 10 non-lesional CAD skin) and 11 healthy control animals. Biopsy of lesional CAD skin showed orthokeratotic hyperkeratosis. Immunohistochemistry (IHC) of Ki-67, a cellular marker for proliferation, showed a higher epidermal cell proliferation rate in the CAD dogs ($p< 0.05$) which positively correlated with Canine Atopic Dermatitis Extent and Severity Index, CADESI-03 ($p< 0.01$). IHC labeling indicated reduced expression of cornified envelope proteins, involucrin (IVL) and filaggrin (FLG) in both lesional and non-lesional skin ($p< 0.05$). In contrast, expression of lympho-epithelial Kazal-type inhibitor (LEKTI), which has been reported to inhibit proteases that cleave proFLG into FLG, was increased in lesional skin compared to the normal controls ($p< 0.05$). The IHC results showed a positive correlation between CADESI-03 and LEKTI expression and a negative correlation between CADESI-03 and IVL expression. In conclusion, the present study demonstrates that epidermal hyperkeratosis in CAD is related to disturbance of both epidermal proliferation and cornified envelope differentiation. This is the first report of a correlation of LEKTI with CAD.

Keywords: canine atopic dermatitis, filaggrin, involucrin, Ki-67, LEKTI

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บทคัดย่อ

การแสดงออกของโปรตีนที่เกี่ยวข้องกับการเพิ่มจำนวนและการเจริญเติบโตของผิวหนังในโรคผิวหนังชนิดอะโทปีในสุนัข

ศิรินทร์ ธีระวัฒนศิริกุล [1] อัจฉริยา ไศละสูต [1] รุ่งโรจน์ ธนาวงษ์นุเวช[1] ทศพร นักเบศร์ [2] คมกริช จ่างแก้ว [3] กรรณาภรณ์ สุริยผล[4]*

จากการศึกษาการเปลี่ยนแปลงทางพยาธิวิทยาของเซลล์คีราติโนไซต์ทั้งในแง่ของการเพิ่มจำนวนเซลล์และการเจริญเติบโตของเซลล์ในสุนัขพันธุ์เล็กที่ป่วยเป็นโรคผิวหนังชนิดอะโทปีโดยการตัดชิ้นเนื้อจากผิวหนังจำนวน 20 ตัว (ผิวหนังส่วนที่มีรอยโรค 10 ตัวอย่างและส่วนที่ไม่มีรอยโรค 10 ตัวอย่าง) เปรียบเทียบกับผิวหนังสุนัขปกติ 11 ตัว พบลักษณะออร์โทคีราโตติคไฮเปอร์คีราโตซิส จากการศึกษาการแสดงออกของโปรตีน Ki-67 ซึ่งแสดงถึงการเพิ่มจำนวนเซลล์ด้วยวิธีอิมมูนโนฮิสโตเคมีพบอัตราการเพิ่มจำนวนเซลล์สูงขึ้นในสุนัขป่วย ($p< 0.05$) และการแสดงออกของโปรตีนดังกล่าวมีความสัมพันธ์เชิงบวกกับดัชนีแสดงความรุนแรงของโรค (CADESI-03) ($p< 0.01$) นอกจากนี้ได้ทำการศึกษาการแสดงออกของโปรตีนในกลุ่มคอร์นิไฟด์เอนเวลโลป (อินโวลูครินและฟิแลกกริน) และโปรตีนเลคติซึ่งทำหน้าที่ยับยั้งโปรติเอสที่ตัดโปรฟิแลกกรินเป็นฟิแลกกรินด้วยวิธีอิมมูนโนฮิสโตเคมี พบการแสดงออกของอินโวลูครินและฟิแลกกรินลดลงทั้งในผิวหนังส่วนที่มีรอยโรคและผิวหนังส่วนที่ไม่มีรอยโรค ($p< 0.05$) ในทางตรงกันข้ามพบการแสดงออกของเลคติเพิ่มขึ้นในผิวหนังส่วนที่มีรอยโรคเมื่อเทียบกับกลุ่มควบคุม ($p< 0.05$) และพบความสัมพันธ์เชิงบวกระหว่าง CADESI 03-และเลคติ และความสัมพันธ์เชิงลบระหว่าง CADESI 03-และอินโวลูคริน จากผลการศึกษาครั้งนี้สามารถสรุปได้ว่า ลักษณะไฮเปอร์คีราโตซิสในผิวหนังสุนัขที่เป็นโรคผิวหนังชนิดอะโทปีเกี่ยวข้องกับทั้งการรบกวนการเพิ่มจำนวนเซลล์และการเจริญเติบโตของเซลล์ การศึกษาครั้งนี้ได้รายงานความสัมพันธ์ระหว่างเลคติและโรคผิวหนังชนิดอะโทปีในสุนัขเป็นครั้งแรก

คำสำคัญ: โรคผิวหนังชนิดอะโทปีในสุนัข ฟิแลกกริน อินโวลูคริน Ki-67 เลคติ

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Introduction

Canine atopic dermatitis (CAD) is the second most common allergic skin disease in dogs (Hillier and Griffin, 2001). CAD prevalence has been increasing in the past decade with approximately 10% of the dog population suffering from the disease (Scott et al., 2001). CAD was found to be associated with impaired epidermal barrier, resulting in increased transepidermal water loss (TEWL) and a defective lamellar lipid bilayer in atopic dogs compared with clinically normal controls (Shimada et al., 2009). The epidermal barrier is formed by epidermal keratinocytes that migrate from the basal layer to the spinous layer, granular layer and eventually to the corneum layer, the outermost layer of the epidermis. The stratum corneum plays a crucial role in the permeability barrier of the skin since hydrating cornified envelop (CE) proteins such as filaggrin and involucrin are formed in this layer. Although the expression levels of FLG and IVL proteins have been reported, their association to CAD is still obscure (Marsella et al., 2009; Chervet et al., 2010). The lymphoepithelial Kazal-type-related inhibitor (LEKTI), encoded by the serine protease inhibitor Kazal-type 5 (SPINK5) gene, is involved in regulation of proteolysis in epithelia formation and keratinocyte terminal differentiation (Chavanas et al., 2000). The LEKTI protein inhibits the protease matriptase, which controls protease activity in the conversion of pro-filaggrin to functional FLG (List et al., 2003; O'Regan et al., 2008). To our knowledge, the association of LEKTI and FLG in CAD has not yet been demonstrated.

Epidermal hyperproliferation indicates a defect of the epidermal barrier function (Proksch et al., 2009). Ki-67, a nuclear protein expressed in cycling cells, is widely used in routine pathology as a cell

proliferation marker in both humans and canines. The Ki-67 antigen is detected within the cell nucleus during interphase. However, during mitosis, it is relocated to the surface of the chromosomes. Ki-67 protein is expressed during the active phases of the cell cycle (G_1, S, G_2, and mitosis), but is absent from postmitotic cells (G_0) (Gerdes et al., 1984). 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 (Olivry et al., 2007).

The aim of this study was to quantify the expression levels of FLG, IVL and LEKTI in lesional atopic, non-lesional atopic and healthy canine skin. The keratinocyte proliferation rate was also studied by Ki-67 expression. The association of the protein expression in atopic skin to the CADESI-03 score was investigated.

Materials and Methods

Animals: Thirty-one dogs were recruited from private small animal clinics in Bangkok, Thailand. Dogs with AD, comprising 12 Poodles, 6 Shih tzus and 2 Pugs, with a mean age of 7 years (age ranged from 2 to 11 years), and the healthy controls, comprising 7 Poodles, 3 Shih tzus and 1 Pug, with a mean age of 7 years (age ranged from 1 to 10 years) were included in this study. 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). This set of criteria comprises age at onset under 3 years, mostly living indoor, corticosteroid-responsive pruritus, chronic or recurrent yeast infections, affected front feet, affected ear pinnae, non-affected ear margins, and non-affected dorso-lumbar area. The International Committee for Allergic Diseases of Animals (or International Task Force on CAD) has recently accepted the concept that food ingredients can cause CAD or, in other words, some dogs with cutaneous adverse food reactions (CAFR or food allergies) might be manifested as CAD, namely the food-induced AD (FIAD). Furthermore, CAFR itself can also show other clinical signs such as hives or pruritus without lesions or with lesions at unusual sites (e.g. flanks, dorsum, perineum, around the lips) (Olivry et al., 2010). Hence, food testing was not the major criterion in sample collection in the present study. Bacterial and yeast infections and ectoparasite infestation were controlled prior to inclusion. The cytology (tape preparation, skin imprint) did not shown bacteria cocci or rods and suppurative inflammation, and round-to-oval budding yeast per high power field (100x) for yeast infection. No anti-inflammatory medication was given for at least 3 weeks prior to examination. 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, excoriations, lichenification, and self induced alopecia) 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. Healthy control samples were taken from clinically normal skin from animals with no history or clinical signs of skin diseases. All animal were used with the consent of the dog owners and following the ethical guidelines required under the 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 another atopic dog group whereas control samples (n= 11) were from clinically normal dogs. A biopsy was taken from each dog after local anesthesia with 2% lidocaine and sutured routinely. Subcutaneous fat was stripped off before each biopsy was bisected. The samples were immersion fixed in 10% neutral buffered formalin for 24 hours, followed by standard tissue processing and paraffin embedding for a routine histopathological and immunohistochemical study.

Histology and Immunohistochemistry: 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 95^0C for 40 min and for the other 4 antigens, the slides were trypsinized by 1.0% trypsin (Merck, Rockland, MA) at 37^0C for 15 min. Endogenous peroxidase was quenched by incubating the slides in 3% hydrogen peroxide in dH_2O for 5 min. Non-specific immunoglobulin binding was blocked with 2% bovine serum albumin at room temperature for 20 min (Merck, Rockland, MA). A mouse monoclonal Ki-67 antibody (MIB-1) (Dako, Glostrup, Denmark) at dilution of 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 filaggrin antibody, a mouse monoclonal involucrin antibody (SY5) (Abcam, Cambridge, UK), and a rabbit polyclonal LEKTI antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were used at dilutions of 1:2000, 1:1000, and 1:3000, respectively. All antibodies were incubated at 4^0C overnight. A Polymer-based nonavidin–biotin system EnVision

(Dako, Glostrup, Denmark) was used for immunolabeling at room temperature for 30 min 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, and LEKTI 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 classifying 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 4 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). 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 division of positive cytoplasmic staining (mm^2) per total area of epidermis (mm^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.

Statistical analysis: Statistical analysis of the thickness of epidermis, the immunohistochemical staining data, and the correlation of determination (R^2) was conducted using a GraphPad Prism software, version 5.0 (San Diego, California). R^2 shows the regression of CADESI scores (the dependent variable) against ratios of Ki-67/mm skin thickness (the independent variable). For protein staining scores, statistical differences were performed by non-parametric Kruskal-Wallis test and Dunn's multiple comparison post test. For thickness of epidermis and of S. corneum, and ratios of Ki67 positive cells per thickness of epidermis (mm), statistical differences were determined using one-way analysis of variance (ANOVA), and significant differences were determined by a Tukey-Kramer test.

Results

Histopathology: The main histopathological feature was epidermal hyperplasia with predominantly orthokeratotic hyperkeratosis. Compared with normal skin which is composed of at least 2-3 well-defined nucleated epidermal layers, the total thickness of lesional and non-lesional epidermis was significantly higher as shown in Table 1. The histopathological finding is shown in Fig 1. Lesional skin demonstrated epidermal hyperplasia with increased desmosomal junction areas and marked accumulation of cells in S. spinosum. Folds penetrating into the dermis were developed. Discrete areas of parakeratosis, hypergranulosis, spongiosis and epidermal edema were observed in lesional skin as well as an aggregation of multifocal microabscesses in superficial epidermis. The dermal change presented edema between collagen bundles and a low-to-mild dermal perivascular inflammatory infiltrate was seen in the lesional skin. The infiltrate was composed of mononuclear cells and a mix of neutrophils, mast cells and eosinophils.

Immunohistochemistry expression of Ki-67, IVL, FLG, and LEKTI in CAD lesional, CAD non- lesional, and normal dog skin: IHC for Ki-67 antigen was confined to nuclei of nucleated epidermal cells, indicating increased germinative cells in the basal epidermis. The hyperplastic proliferation rate in epidermis of skin was found to be enhanced in non-lesional and more pronounced in lesional skin as shown by the increased staining scores of Ki-67 proliferative activity (Fig 2A-D). The result correlated with the lesional appearance of the skin.

Table 1 Thickness of epidermis and ratio of S. corneum and nucleated epidermis of non-lesional and lesional skin of dogs with AD

	Normal	CAD non-lesion	CAD lesion
Thickness of S. corneum (μm)	7.746±2.535[a]	11.879±3.242[b]	34.857±9.106[c]
Thickness of nucleated epidermis (μm)	14.818±2.271[a]	24.250±5.940[b]	137.214±40.210[c]
Ratio of S. corneum and nucleated epidermis	0.493±0.132[a]	0.568±0.255[a]	0.273±0.080[b]

The superscript letters are significantly different (one way ANOVA at $p< 0.05$).

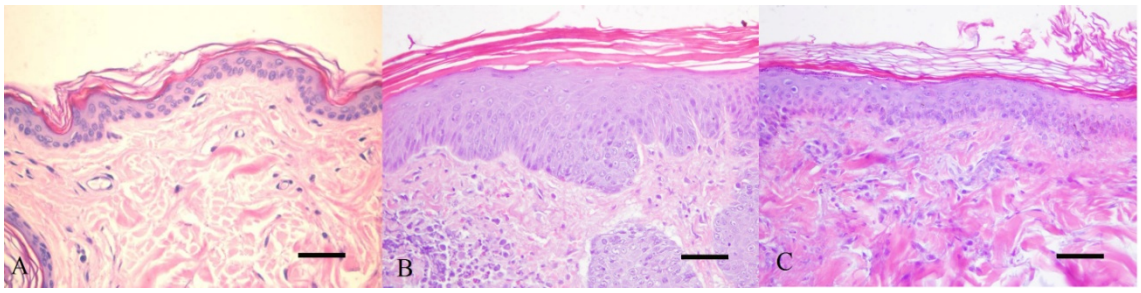

Figure 1 Histopathology of normal clinical (A), CAD lesional (B) and non-lesional skins (C) (scale bar in *A* = 20µm; scale bars in B and C = 50 µm)

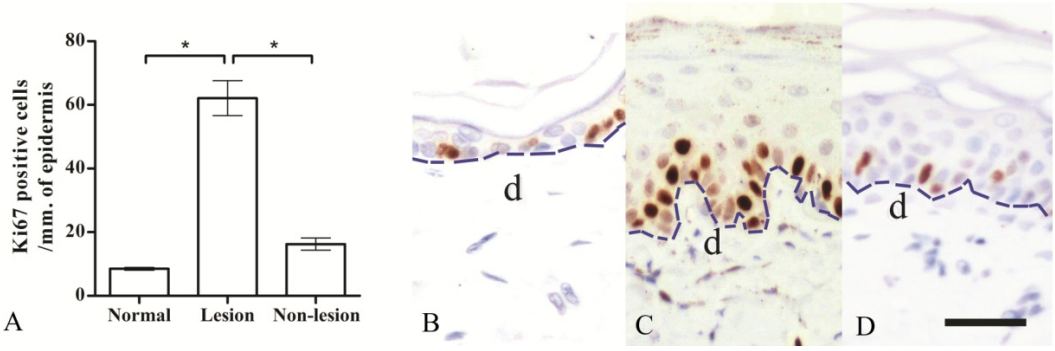

Figure 2 Immunohistochemical staining for Ki-67. (A) Ki-67-positive cells in normal, lesional and non-lesional skins. Bars indicate mean±SD data with significant *p*-values *p< 0.05. Protein expression of Ki-67 in normal (scale bars= 30 µm), lesional (scale bars= 50 µm), and non-lesional skins (scale bars= 50 µm) (Fig 2B-D, respectively) d: dermis.

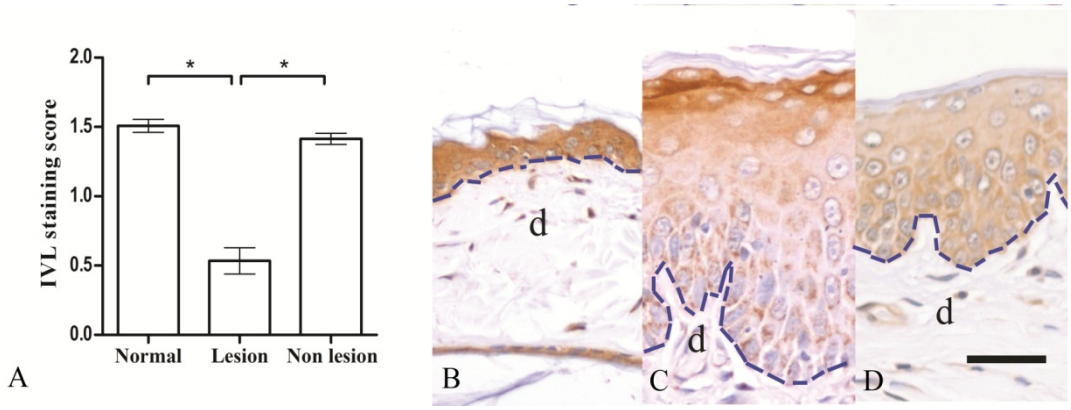

Figure 3 Immunohistochemical staining for IVL. (A) IVL staining scores in normal, lesional and non-lesional skins. Bars indicate mean±SD data with significant *p*-values *p< 0.05. Protein expression of IVL in normal (scale bars= 30 µm), lesional (scale bars= 50 µm), and non-lesional skins (scale bars= 50 µm) (Fig 3B-D, respectively) d: dermis.

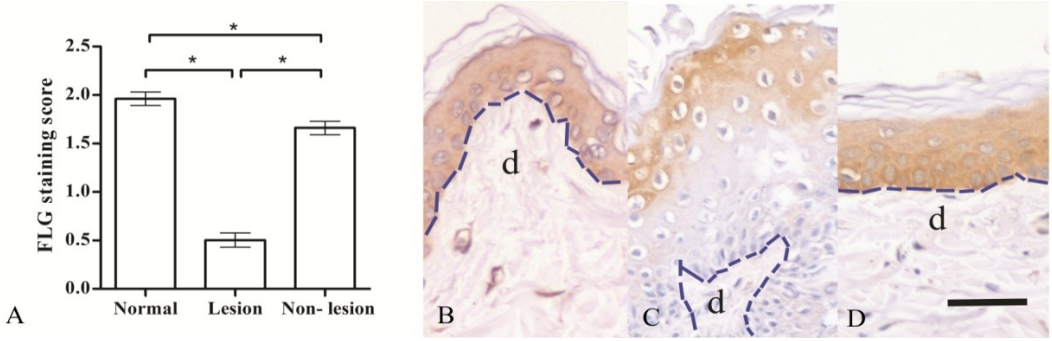

Figure 4 Immunohistohemical staining for FLG. (A) FLG staining scores in normal, lesional and non-lesional skins. Bars indicate mean±SD data with significant *P*-values *p< 0.05. Protein expression of FLG in normal (scale bars= 30 µm), lesional (scale bars= 50 µm), and non-lesional skins (scale bars= 50 µm) (Fig 4B-D, respectively) d: dermis.

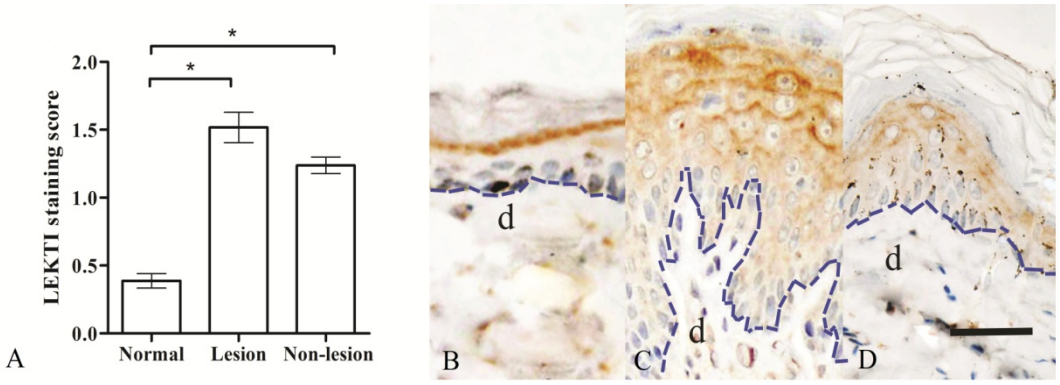

Figure 5 Immunohistohemical staining for LEKTI. (A) LEKTI staining scores in normal, lesional and non-lesional skins. Bars indicate mean±SD data with significant *p*-values *$p< 0.05$. Protein expression of LEKTI in normal (scale bars= 30 µm), lesional (scale bars= 50 µm), and non-lesional skins (scale bars= 50 µm) (Fig 5B-D, respectively) d: dermis.

The expression of IVL and FLG, marker proteins of terminal epidermal differentiation was analyzed. IVL expression was observed (brown to deep brown granules in Fig 3A-D) in the entire nucleated epidermal layers and the lower part of S. corneum in normal skin. The intensity was significantly decreased in lesional skin. An irregular and discontinuous expression pattern of IVL with variable epidermal cell layers of S. granulosum and S. spinosum was observed in CAD skin. Decreased to absent expression was observed in the lower part of the S. spinosum and basal layers. In the S. granulosum, which was markedly infiltrated by inflammatory cells and displayed spongiotic areas, the expression levels were barely detectable (Fig 3A-D, Table 2). FLG immunohistochemical staining was observed in the entire nucleated epidermis. Similarly to IVL, FLG expression was significantly reduced in lesional ($p< 0.05$). The lesion showed very weak intensity staining in the lower part of S. granulosum, S. spinosum and S. basale, whereas discontinuous staining was revealed in the upper part of S. granulosum and S. corneum (Fig 4A-D, Table 2).

LEKTI was obviously expressed as a band in S.corneum of normal skin with moderate to strong intensity. In contrast, owing to epidermal hyperplasia in lesional skin, LEKTI was expressed in variable degree from the lower part of S. corneum to the upper part of S. spinosum. In spongiotic and parakeratotic areas, the staining intensity was very strong. The staining in non-lesional skin was predominantly at the S. corneum and S. granulosum and there was neither spongiosis nor parakeratosis. The IHC staining scores for CAD were significantly increased in non-lesional and more pronounced in lesional skin compared to the controls ($p< 0.05$) (Fig 5A-D, Table 2).

The protein expression in both lesional and non-lesional samples was compared with clinical severity scores CADESI-03. Ki-67 staining nuclei of both lesional and non-lesional skins was shown to be significantly positively correlated with clinical severity scores ($p< 0.01$, $R^2= 0.70$; $p< 0.01$, $R^2= 0.64$, respectively) (Fig 6A-B). IVL staining scores, on the other hand, were shown to be significantly negatively correlated with the severity scales ($p< 0.001$, $R^2= 0.62$; $p< 0.05$, $R^2= 0.51$, respectively) (Fig 7A-B). Finally, LEKTI staining scores of lesional skins were also shown to be significantly positively correlated with clinical severity scores ($p< 0.05$, $R^2= 0.58$) (Fig 7C).

Discussion

In this study, we present an analysis of CAD histopathology and show an alteration of the expression patterns of proteins Ki-67, IVL, FLG and SPINK5, which are involved in epidermal proliferation and differentiation in CAD. The microscopic changes in lesional CAD skin described in the present study, such as orthokeratotic hyperkeratosis, resemble the results of previous reports showing epidermal hyperplasia, hyperkeratosis, hypergranulosis and spongiosis (Yager and Wilcock, 1994; Olivry et al., 1997; de Mora et al., 2007). Erythema, one of the major markers of primary lesions, was found in 6 AD dogs whereas four dogs showed lichenification, one of the major markers of secondary lesions. The CAD histology

Table 2 IHC staining scores of IVL, FLG and LEKTI expression by semi-quantitative manual scoring for normal, non-lesional and lesional skin of CAD

	Normal	CAD non-lesional skin	CAD lesional skin
IVL	++/+++ D	+/+++ C-D	0/++ A-B
FLG	++/+++ D	+/+++ C-D	0/+++ A-B
LEKTI	++/+++ A	++/+++ B-C	++/++ B-C

Semi-quantitative manual scoring; staining intensity 0, negative; +, mild; ++, moderate; +++, strong. A, low proportion (<25%); B, moderate proportion (25-50%); C, high proportion (50-75%); D, almost entire proportion (more than 75%) are positive of epidermis.

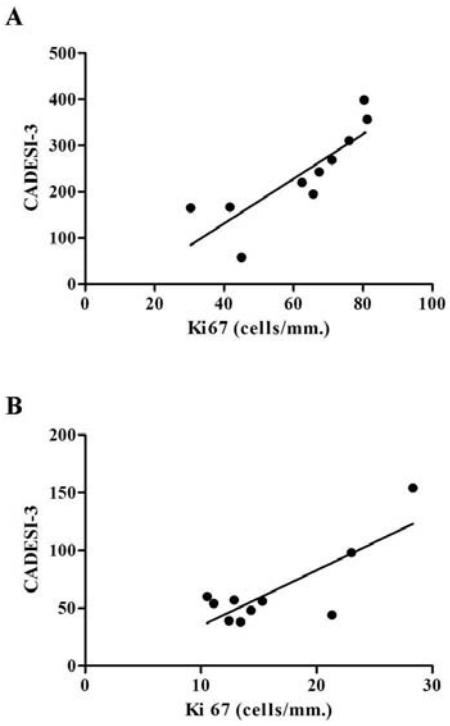

Figure 6 Correlation between CADESI-03 and Ki-67 staining nuclei in lesional and non-lesional skin of dogs with AD. Association of CADESI-03 and Ki-67 staining nuclei in lesional and non-lesional skins is presented (Fig 6A-B, respectively).

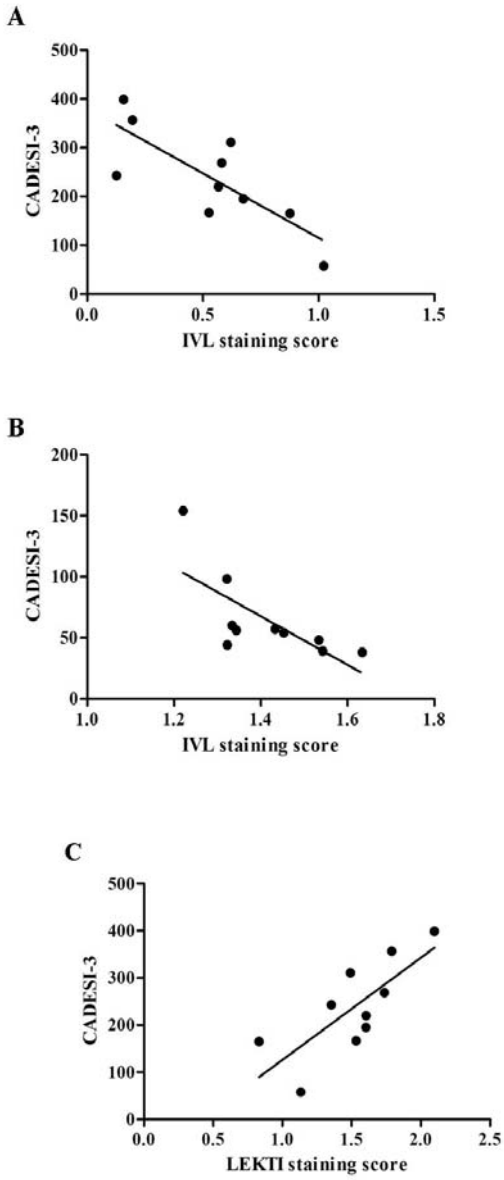

Figure 7 Correlation between CADESI-03 K5 and IVL staining scores in lesional and non-lesional skin of dogs with AD. Association of CADESI-03 and IVL staining scores in lesional and non-lesional skins (Fig 7A-B, respectively); Association of CADESI-03 and LEKTI staining scores in lesional skin (Fig 7C).

was similar to that of human chronic AD (Leung, 1999). However, there were no pathognomonic lesions of CAD since there were several confounding factors such as variations in the environment, diet, genetics, and age of individual lesions. Furthermore, histopathology often serves only to rule out some of the clinical different diagnosis. Lesions are no more than supportive of a tentative clinical diagnosis (Yager and Wilcock, 1994; Marsella and Olivry, 2003). In addition, we observed similar skin-infiltrating cells (mononuclear cells, neutrophils, mast cells and eosinophils) to other studies (Yager and Wilcock, 1994; Olivry et al., 1997; Scott et al., 2001; de Mora et al., 2007). The difference in the cellular infiltrate probably results from variations in the immune reaction between single and repeated allergen exposure as well as epidermal versus dermal antigen contact.

Antigen Ki-67 is a nuclear protein related to cell proliferation and is expressed in cell nuclei throughout the entire active phases of the cell cycle (G_1, S, G_2, and mitosis), but is absent from resting cells (G_0). Therefore, there is a widely accepted proliferation marker of cell division (Scholzen and Gerdes, 2000). The significantly increased expression of Ki-67 in atopic skin implied that epidermal hyperplasia in lesional CAD skin was also associated with an accumulation of cells. This hyperproliferation-associated hyperplasia is in agreement with earlier studies in human atopic dermatitis (HAD) skin, which was shown to be highly proliferated (Sapuntsova et al., 2002; Bovenschen et al., 2005). Hyperproliferation is probably an attempt to reconstitute normal barrier function and to remove invading antigens (Jensen et al., 2004). In dogs, generally after skin irritation or superficial trauma of epidermis, the mitotic activity of basal epidermal cells is induced to generate new cell population which results in epidermal hyperplasia within 36-48 hr (Yager and Wilcock, 1994). The correlations of Ki-67 staining scores with CADESI in the present study suggest an association between hyperproliferation-associated hyperplasia and disease severity.

Hyperproliferation is usually accompanied by disturbed differentiation, probably due to insufficient cell differentiation time, a critical period

for the permeability barrier formation (Ekanayake-Mudiyanselage et al., 1998; Jensen et al., 2004). In the present study, the alteration of protein expression associated with epidermal differentiation was demonstrated.

IVL staining was observed in the entire nucleated epidermal layers and down to the lower horny layer in normal dog skin. Human IVL has been shown to be normally expressed in upper spinous layers and the S. granulosum (Jensen et al., 2004; Proksch et al., 2009). We found that it was difficult to differentiate immunostained cell layers in healthy dog skin because canine skin (10-30 µm) is thinner than that of human (50-100 µm) (Lloyd and Garthwaite, 1982; Kanistakis, 2002). However, similar to HAD, IVL protein expression was significantly decreased in CAD skin ($p< 0.05$) although the immunostaining band of atopic skin was broader. Reduced IVL expression in the S. spinosum of HAD patients resulted in fewer ceramides in the S. corneum (Jensen et al., 2004; Jarzab et al., 2010). The IVL expression band was expanded to the lower spinous layer in both non-lesional and lesional skin (Jensen et al., 2004). An inverse correlation between IVL staining scores and CADESI-03 disease severity scores strongly supports a role for IVL in CAD. This result contradicts a previous study that showed an aberrant increase of IVL expression in CAD (Chervet et al., 2010).

During epidermal differentiation, FLG and IVL play a crucial role in the formation of the CE and the establishment of epidermal barrier function (Watt, 1983; Steven and Steinert, 1994). There have been several studies of FLG immunostaining in dogs. Bardagi et al. (2007) showed the expression of filaggrin in cytoplasmic and keratohyalin granules in the epidermal granular layer in normal dog skin. In our study, FLG staining in normal skin was found in all epidermal layers, due to the very thin layers of dog skin as mentioned above. We also showed that the FLG staining in atopic dogs was significantly reduced compared to the controls. This result corresponds to a reduced FLG expression in 39% (7 of 18) of dogs with AD compared with that of controls when using the N- and C-terminal FLG antibodies (Chervet et al., 2010) and in laboratory beagles, with induced CAD (Marsella et al., 2009). In addition, decreased FLG and IVL immunostaining was remarkably found in HAD skin (Cline and Rice, 1983; Seguchi et al., 1996; Ekanayake-Mudiyanselage et al., 1998; Jensen et al., 2004). The increased protein expression of IVL and FLG was in contrast to the upregulation of their mRNAs observed in our previous study (Theerawatanasirikul et al., 2012). However, the correlation between mRNA levels and the expression of the corresponding proteins is not entirely clear since several studies have demonstrated different results between mRNA and protein expressions (Gygi et al., 1999; Chen et al., 2002; Ji et al., 2003). The upregulation of mRNA is probably a compensatory mechanism in response to the decreased protein expression in AD skin. In addition, the lack of correlation between mRNA and protein levels may be the indicative of processing acting at the levels of translation or post-translational modification and it may be due to the number of isoforms for each gene (Chen et al., 2002; Fournier et al., 2010). For example, the mutation of a gene may lead to reduced protein expression without any effect on the transcription level (e.g. *FLG* gene in HAD) (Palmer et al., 2006; Cork et al., 2009).

In the present study, the expression of the serine protease inhibitor LEKTI protein expression was found to be significantly increased in CAD lesional samples. LEKTI inhibits the protease matriptase, which cleaves proFLG to produce the active FLG (List et al., 2003; O'Regan et al., 2008; Cork et al., 2009). The balance between proteases and protease inhibitors determines the rate of desquamation and also skin thickness (Cork et al., 2009). In humans, the link between LEKTI protein expression and HAD is still unclear since it has been reported that a mutation of the *SPINK5* gene, leading to low levels of LEKTI, is not associated with the disease (Hubiche et al., 2007). In this study, LEKTI expression was found to be increased in CAD and LEKTI staining scores were also significantly correlated with the clinical severity scores. This is in agreement with the decreased FLG expression. We, therefore, hypothesize that a higher concentration of LEKTI protein will result in reduced protease activity, leading to the accumulation of the proFLG form.

In conclusion, this study reveals, at least in part, the pathology of CAD at the protein level. The hyperproliferation of keratinocytes was demonstrated by Ki-67 protein staining and the defective differentiation was associated with a lower expression of IVL and FLG, and a higher expression of LEKTI. The next step should be the investigation of the expression levels of other proteases and protease inhibitors.

References

Bardagi, M., Vala, H., Fondevila, D. and Ferrer, L. 2007. Immunohistochemical detection of filaggrin in the skin of different species. Vet Dermatol. 18: 388.

Bovenschen, H.J., Seyger, M.M. and Van de Kerkhof, P.C. 2005. Plaque psoriasis vs. atopic dermatitis and lichen planus: a comparison for lesional T-cell subsets, epidermal proliferation and differentiation. Br J Dermatol. 153: 72-78.

Chavanas, S., Bodemer, C., Rochat, A., Hamel-Teillac, D., Ali, M., Irvine, A.D., Bonafé, J.L., Wilkinson, J., Taïeb, A., Barrandon, Y., Harper, J.I., de Prost, Y. and Hovnanian, A. 2000. Mutations in SPINK5, encoding a serine protease inhibitor, cause Netherton syndrome. Nat Genet. 25: 141-142.

Chen, G., Gharib, T.G., Huang, C.C., Taylor, J.M., Misek, D.E., Kardia, S.L., Giordano, T.J., Iannettoni, M.D., Orringer, M.B., Hanash, S.M. and Beer, D.G. 2002. Discordant protein and mRNA expression in lung adenocarcinomas. Mol Cell Proteomics. 1: 304-313.

Chervet, L., Galichet, A., McLean, W.H., Chen, H., Suter, M.M., Roosje, P.J. and Müller, E.J. 2010. Missing C-terminal filaggrin expression,

NFkappaB activation and hyperproliferation identify the dog as a putative model to study epidermal dysfunction in atopic dermatitis. Exp Dermatol. 19: e343-e346.

Cline, P.R. and Rice, R.H. 1983. Modulation of involucrin and envelope competence in human keratinocytes by hydrocortisone, retinyl acetate, and growth arrest. Cancer Res. 43: 3203-3207.

Cork, M.J., Danby, S.G., Vasilopoulos, Y., Hadgraft, J., Lane, M.E., Moustafa, M., Guy, R.H., Macgowan, A.L., Tazi-Ahnini, R. and Ward, S.J. 2009. Epidermal barrier dysfunction in atopic dermatitis. J Invest Dermatol. 129: 1892-1908.

de Mora, F., de la Fuente, C., Jasmin, P., Gatto, H., Marco, A., Ferrer, L., Fondati, A., Fondevila, D. and Torres, R. 2007. Evaluation of the expression of P-selectin, ICAM-1, and TNF-alpha in bacteria-free lesional skin of atopic dogs with low-to-mild inflammation. Vet Immunol Immunopathol. 115: 223-229.

Ekanayake-Mudiyanselage, S., Aschauer, H., Schmook, F.P., Jensen, J.M., Meingassner, J.G., Proksch, E. 1998. Expression of epidermal keratins and the cornified envelope protein involucrin is influenced by permeability barrier disruption. J Invest Dermatol. 111: 517-523.

Favrot, C., Steffan, J., Seewald, W. and Picco, F. 2010. A prospective study on the clinical features of chronic canine atopic dermatitis and its diagnosis. Vet Dermatol. 21: 23-31.

Fournier, M.L., Paulson, A., Pavelka, N., Mosley, A.L., Gaudenz, K., Bradford, W.D., Glynn, E., Li, H., Sardiu, M.E., Fleharty, B., Seidel, C., Florens, L. and Washburn, M.P. 2010. Delayed correlation of mRNA and protein expression in rapamycin-treated cells and a role for Ggc1 in cellular sensitivity to rapamycin. Mol Cell Proteomics. 9: 271-284.

Gerdes, J., Lemke, H., Baisch, H., Wacker, H.H., Schwab, U. and Stein, H. 1984. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol. 133: 1710-1715.

Gygi, S., Rochon, Y., Franza, B.R. and Aebersold, R. 1999. Correlation between protein and mRNA abundance in yeast. Mol Cell Biol. 19: 1720-1730.

Hillier, A. and Griffin, C.E. 2001. The ACVD task force on canine atopic dermatitis (I): incidence and prevalence. Vet Immunol Immunopathol. 81: 147-151.

Hubiche, T., Ged, C., Benard, A., Léauté-Labrèze, C., McElreavey, K., de Verneuil, H., Taïeb, A. and Boralevi, F. 2007. Analysis of SPINK 5, KLK 7 and FLG genotypes in a French atopic dermatitis cohort. Acta Derm Venereol. 87: 499-505.

Jarzab, J., Filipowska, B., Zebracka, J., Kowalska, M., Bozek, A., Rachowska, R., Gubala, E., Grzanka, A., Hadas, E. and Jarzab, B. 2010. Locus 1q21 gene expression changes in atopic dermatitis skin lesions: deregulation of small proline-rich region 1A. Int Arch Allergy Immunol. 151: 28-37.

Jensen, J.M., Folster-Holst, R., Baranowsky, A., Schunck, M., Winoto-Morbach, S., Neumann, C., Schütze, S. and Proksch, E. 2004. Impaired sphingomyelinase activity and epidermal differentiation in atopic dermatitis. J Invest Dermatol. 122: 1423–1431.

Ji, P., Xuan, J.W., Onita, Y., Sakai, H., Kanetake, H., Gabril, M.J., Sun, Y., Moussa, M. and Chin, JL. 2003. Correlation study showing no concordance between EPAS-1/HIF-2alpha mRNA and protein expression in transitional cell cancer of the bladder. Urology. 61: 851-857.

Kanitakis, J. 2002. Anatomy, histology and immunohistochemistry of normal human skin. Eur J Dermatol. 12: 390-401.

Leung, D.Y. 1999. Pathogenesis of atopic dermatitis. J Allergy Clin Immunol. 104: S99–S108.

List, K., Szabo, R., Wertz, P.W., Segre, J., Haudenschild, C.C., Kim, S.Y. and Bugge, T.H. 2003. Loss of proteolytically processed filaggrin caused by epidermal deletion of Matriptase/MT-SP1. J Cell Biol. 163: 901-910.

Lloyd, D.H. and Garthwaite, G. 1982. Epidermal structure and surface topography of canine skin. Res Vet Sci. 33: 99-104.

Marsella, R. and Olivry, T. 2003. Animal models of atopic dermatitis. Clin Dermatol. 21: 122-133.

Marsella, R., Samuelson, D. and Harrington, L. 2009. Immunohistochemical evaluation of filaggrin polyclonal antibody in atopic and normal beagles. Vet Dermatol. 20: 547-554.

Olivry, T. 2010. New diagnostic criteria for canine atopic dermatitis. Vet Dermatol. 21: 123-126.

Olivry, T., DeBoer, D.J., Favrot, C., Jackson, H.A., Mueller, R.S., Nuttall, T., Prélaud P; International Task Force on Canine Atopic Dermatitis. 2010. Treatment of canine atopic dermatitis: 2010 clinical practice guidelines from the International Task Force on Canine Atopic Dermatitis. Vet Dermatol. 21: 233-248.

Olivry, T., Marsella, R., Iwasaki, T. and Mueller, R. 2007. Validation of CADESI-03, a severity scale for clinical trials enrolling dogs with atopic dermatitis. Vet Dermatol. 18: 78-86.

Olivry, T., Naydan, D.K. and Moore, P.F. 1997. Characterization of the cutaneous inflammatory infiltrate in canine atopic dermatitis. Am J Dermatopathol. 19: 477-486.

O'Regan, G.M., Sandilands, A., McLean, W.H. and Irvine, A.D. 2008. Filaggrin in atopic dermatitis. J Allergy Clin Immunol. 122: 689-693.

Palmer, C.N., Irvine, A.D., Terron-Kwiatkowski, A., Zhao, Y., Liao, H., Lee, S.P., Goudie, D.R., Sandilands, A., Campbell, L.E., Smith, F.J., O'Regan, G.M., Watson, R.M., Cecil, J.E., Bale, S.J., Compton, J.G., DiGiovanna, J.J., Fleckman, P., Lewis-Jones, S., Arseculeratne, G., Sergeant, A., Munro, C.S., El Houate, B., McElreavey, K., Halkjaer, L.B., Bisgaard, H., Mukhopadhyay, S. and McLean, W.H. 2006. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. Nat Genet. 38: 441-446.

Proksch, E., Fölster-Holst, R., Bräutigam, M., Sepehrmanesh, M., Pfeiffer, S. and Jensen, J.M. 2009. Role of the epidermal barrier in atopic dermatitis. J Dtsch Dermatol Ges. 7: 899-910.

Sapuntsova, S.G., Mel'nikova, N.P., Deigin, V.I., Kozulin, E.A. and Timoshin, S.S. 2002. Proliferative processes in the epidermis of patients with atopic dermatitis treated with thymodepressin. Bull Exp Biol Med. 133: 488-490.

Scholzen, T. and Gerdes, J. 2000. The Ki-67 protein: from the known and the unknown. J Cell Physiol. 182: 311-322.

Scott, D.W., Miller, W.H. and Griffin, C.E. 2001. Skin immune system and allergic skin diseases. In: Muller & Kirk's Small Animal Dermatology. 6th ed. G.H. Muller, R.W. Kirk, D.W. Scott and C.E. Griffin (eds.). Philadelphia: Saunders: 543-666.

Seguchi, T., Cui, C.Y., Kusuda, S., Takahashi, M., Aisu, K. and Tezuka, T. 1996. Decreased expression of filaggrin in atopic skin. Arch Dermatol Res. 288: 442-446.

Shimada, K., Yoon, J.S., Yoshihara, T., Iwasaki, T. and Nishifuji, K. 2009. Increased transepidermal water loss and decreased ceramide content in lesional and non-lesional skin of dogs with atopic dermatitis. Vet Dermatol. 20: 541-546.

Steven, A.C. and Steinert, P.M. 1994. Protein composition of cornified cell envelopes of epidermal keratinocytes. J Cell Sci. 107: 693-700.

Theerawatanasirikul, S., Sailasuta, A., Thanawongnuwech, R. and Suriyaphol, G. 2012. Alterations of keratins, involucrin and filaggrin gene expression in canine atopic dermatitis. Res Vet Sci. doi: 10.1016/j.rvsc.2012.06.005, in press.

Watt, F.M. 1983. Involucrin and other markers of keratinocyte terminal differentiation. J Invest Dermatol. 81: 100S–103S.

Yager, Y.A. and Wilcock, B.P. 1994. Part II Inflammatory and degenerative lesion of the skin classified by pattern. In: Color Atlas and Text of Surgical Pathology of the Dog and Cat: Dermatopathology and Skin Tumors. St. Louis: Mosby-Year book: 51-139.