standard curves was verified by the R^2 coefficient. The efficiency of the assay was between 92% and 131% and the R^2 value was >0.97.

Statistical qnalysis

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. For the real-time PCR results, the data were analyzed in REST 384 software, using a pair wise fixed reallocation randomization test to test for significance between groups. The results with *P* value <0.05 were considered relevant significance.

4. Results

Histopathology

The main histopathological feature was epidermal hyperplasia with predominantly orthokeratotic hyperkeratosis. Compared with normal skins which 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 3. The histopathological finding was shown in Fig 1. Lesional skins demonstrated epidermal hyperplasia with increased desmosomal junction areas and marked accumulation of cells in S. spinosum. Folds penetrating into dermis were developed. Focal parakeratosis, hypergranulosis, spongiosis and epidermal edema were irregularly appeared in lesional skins together with the 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 by mononuclear cells and a mix of neutrophils, mast cells and eosinophils.

Immunohistochemistry expression of Ki-67, K5, K10, 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 hyperproliferation and hyperplasia indexes as well as the increased staining scores of Ki-67 proliferative activity (Fig 2A-C, Table 4). The result correlated with the lesional appearance of the skin.

Whereas in normal dog skin, K5 is exclusively synthesized by basal cells, in lesional and non-lesional skins, the staining of K5 extended to suprabasal compartment with reduced staining intensity. In non-lesional skins, the staining was mainly concentrated in

Table 3 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 significant different (one way ANOVA at $P < 0.05$).

Fig. 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)

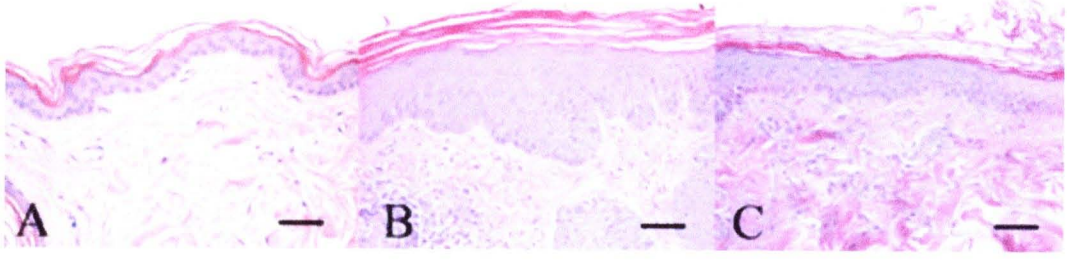


Fig. 2 Immunohistochemical staining for Ki-67, K5, K10, IVL, FLG and LEKTI. (a) Ki-67-positive cells and K5, K10, IVL, FLG and LEKTI staining scores in normal, lesional and non-lesional skins. Bars indicate mean ± SD data with significant *P*-values **P* <0.05. (b) Protein expression of Ki-67, K5, K10, IVL, FLG and LEKTI in normal (at x100 magnification, scale bars = 20 μm) (*A, D, G, J, M, P*), lesional (at x200 magnification, scale bars = 50 μm) (*B, E, H, K, N, Q*) and non-lesional skins (at x200 magnification, scale bars = 50 μm) (*C, F, I, L, O, R*).

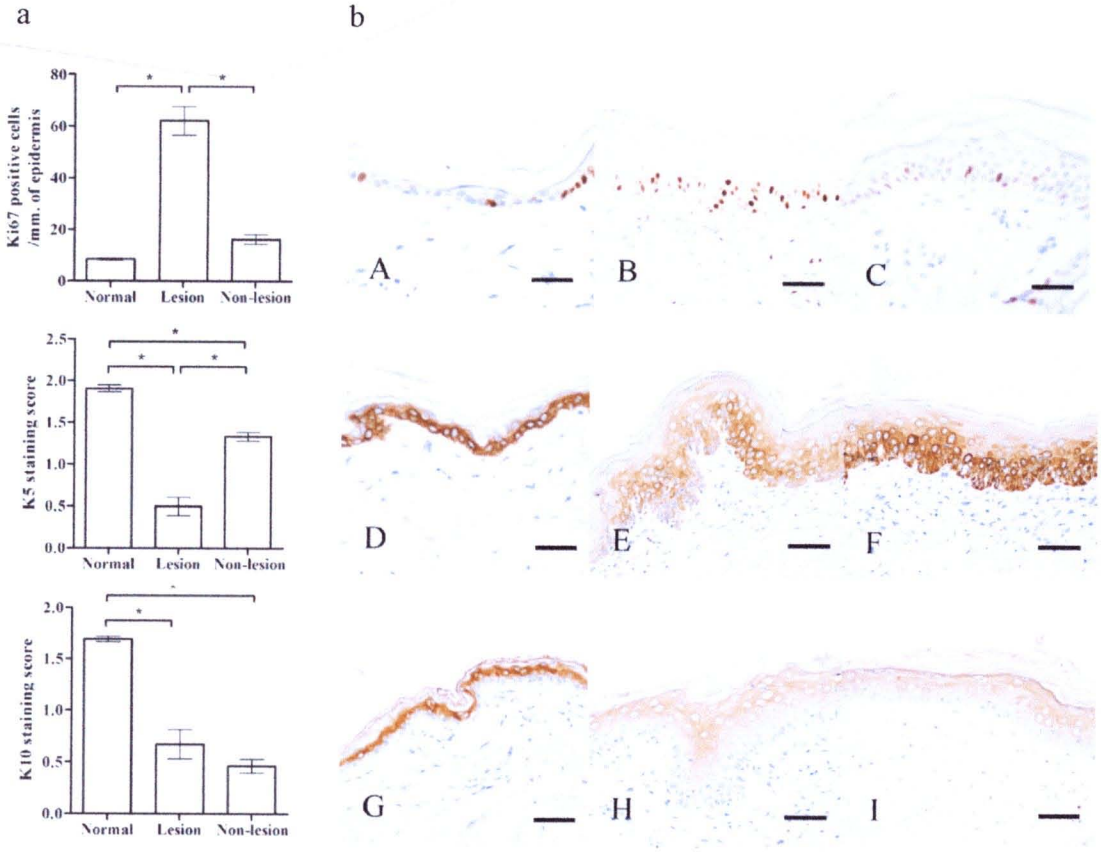


Fig. 2 Continued

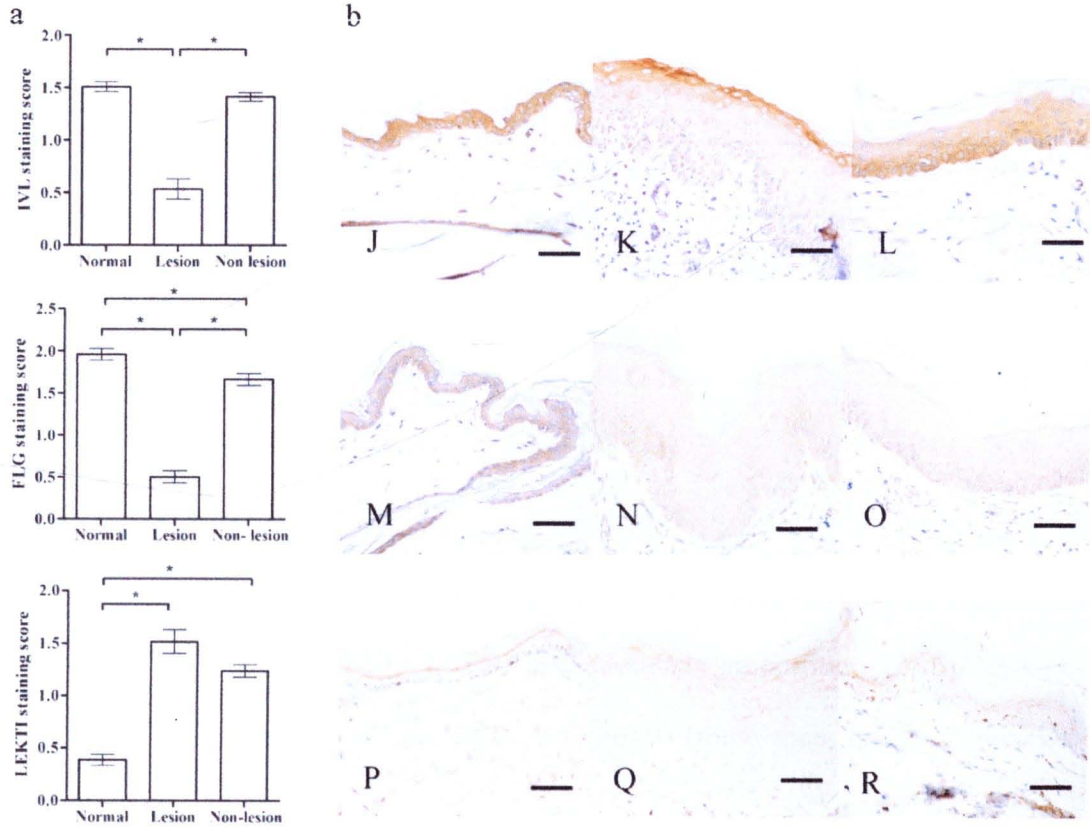


Table 4 IHC staining scores of K5, K10, IVL, FLG expression by semi-quantitative manual scoring for normal, non-lesional and lesional skin of CAD

	Normal	CAD non-lesional skin	CAD lesional skin
	Manual score	Manual score	Manual score
K5	+++ C	++/+++ C-D	+/+++ C-D
K10	+++ B	++/+++ C-D	+/+++ B-D
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.

basal layers but the extension to suprabasal epidermis with mild to moderated expression occurred. In lesional skins, expression was in S. basale and S. spinosum with a significantly reduced intensity compared with normal skins. K5 expression was observed in the areas that corresponding to low K10 staining. The IHC staining scores for CAD were decreased in non-lesional and much less in lesional skins compared to the controls (Fig 2D-F, Table 4).

The differentiation-associated K10 was expressed throughout the suprabasal compartment in normal skins whilst in non-lesional and lesional skin, K10, although less intensive, was found primarily within the uppermost suprabasal cell layers, in S. corneum and the upper part of S. granulosum. The IHC staining scores for CAD were decreased in lesional and non-lesional skins compared to the controls. However, there was no significant difference between non-lesional and lesional skins by image analysis ($P > 0.05$) (Fig 2G-I, Table 4).

The expression of IVL and FLG, marker proteins of early and late epidermal differentiation, respectively (Fuchs 1990), was analyzed. IVL was observed with a brown to deep brown, granular like appearance in the entire nucleated epidermal layers to the lower part of S. corneum in normal skins. The intensity was significantly decreased in lesional skin. Irregular and discontinuous expression pattern with variable epidermal cell layers of S. granulosum and S. spinosum was observed. Decreased to absent intensity was revealed in the lower part of S. spinosum and basal layers whereas in granulosum markedly with inflammatory cell infiltration and spongiotic areas, the staining was almost disappeared (Fig 2J-L, Table 4). FLG immunohistochemical staining appeared in the entired nucleated epidermis. FLG expression was significantly reduced in lesional skin similar to IVL ($P < 0.05$). The lesion showed very weak intensity 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 2M-O, Table 4).

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 skins, LEKTI

was expressed with variable degrees in 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 skins was predominantly at the S. corneum and S. granulosum without spongiosis and parakeratosis. The IHC staining scores for CAD were significantly increased in non-lesional and more pronounced in lesional skins compared to the controls ($P < 0.05$).

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 3A-B) similar to those of K5 ($P < 0.01$, $R^2 = 0.70$; $P < 0.05$, $R^2 = 0.54$, respectively) (Fig 4A-B), whereas IVL staining scores 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 4C-D). Furthermore, LEKTI staining scores of lesional skins were shown to be significantly positively correlated with clinical severity scores ($P < 0.05$, $R^2 = 0.58$) (Fig 4E).

Gene expression in CAD lesional, CAD non-lesional and healthy dog skin

K5, IVL and FLG showed distinct increases in mRNA in lesional skin and between non-lesional and lesional skin. Merely *K5* showed a statistically significant difference in expression between non-lesional and control skin but with a lesser expression than in lesional skin. *K10* expression tended to be upregulated in lesional skin compared to the

Fig. 3 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. 3A-B, respectively).

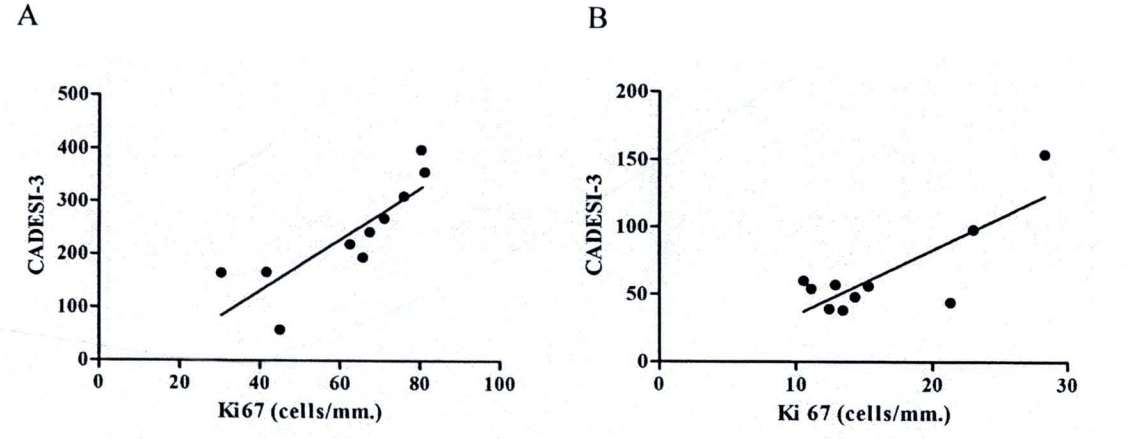


Fig. 4 Correlation between CADESI-03 K5 and IVL staining scores in lesional and non-lesional skin of dogs with AD. Association of CADESI-03 and K5 staining scores in lesional and non-lesional skins (Fig. 4A-B, respectively); Association of CADESI-03 and IVL staining scores in lesional and non-lesional skins (Fig. 4C-D, respectively); Association of CADESI-03 and LEKTI staining scores in lesional skin (Fig. 4E).

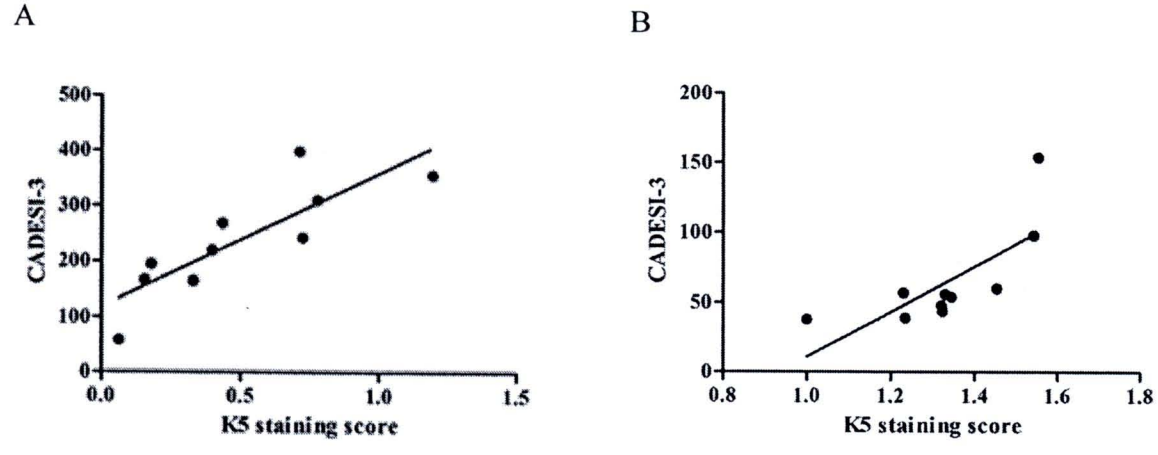
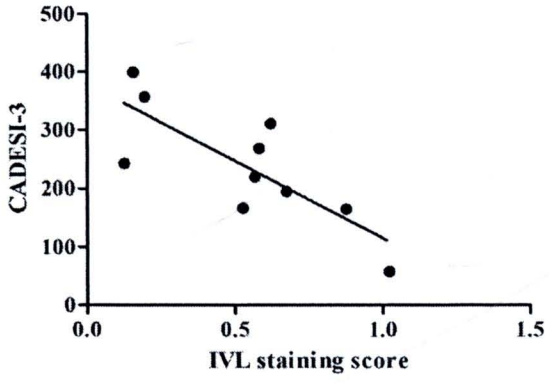


Fig. 4 (Continued)

C



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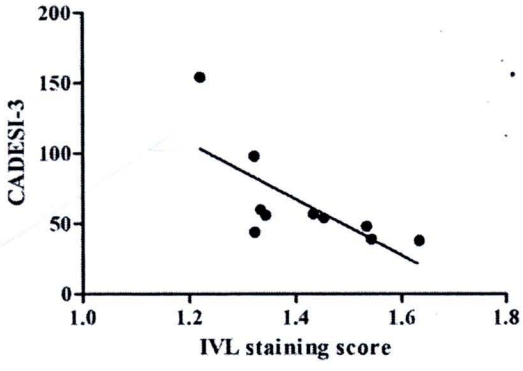

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