Comparison of cellular location and expression of Plakophilin-2 in epidermal cells from nonlesional atopic skin and healthy skin in German shepherd dogs

Brita Ardesjö-Lundgren*, Katarina Tengvall†, Kerstin Bergvall§, Fabiana H.G. Farias*, Liya Wang‡, Ake Hedhammar§, Kerstin Lindblad-Toh‡‡‡ and Göran Andersson†

*Science for Life Laboratory, Department of Medical Biochemistry and Microbiology, BMC, Uppsala University, Box 582, SE-75123 Uppsala, Sweden
†Department of Animal Breeding and Genetics, Swedish University of Agricultural Sciences, Box 7023, SE-75007 Uppsala, Sweden
‡Neuroimmunology Unit, Centrum for Molecular Medicine, Department of Clinical Neuroscience, Karolinska Institutet, 17176 Stockholm, Sweden
§Department of Clinical Sciences, Swedish University of Agricultural Sciences, Box 7054, SE-75007 Uppsala, Sweden
¶Department of Anatomy, Physiology and Biochemistry, Swedish University of Agricultural Sciences, Box 7011, SE-75007 Uppsala, Sweden
**Broad Institute of MIT and Harvard, 415 Main St, Cambridge, MA 02142, USA
††Kerstin Bergvall
§§§Kerstin Lindblad-Toh

Correspondence: Katarina Tengvall, Science for Life Laboratory, Department of Medical Biochemistry and Microbiology, BMC, Uppsala University, Box 582 SE-75123, Uppsala, Sweden. E-mail: katarina.tengvall@ki.se

Background – Canine atopic dermatitis (CAD) is an inflammatory and pruritic allergic skin disease caused by interactions between genetic and environmental factors. Previously, a genome-wide significant risk locus on canine chromosome 27 for CAD was identified in German shepherd dogs (GSDs) and Plakophilin-2 (PKP2) was defined as the top candidate gene. PKP2 constitutes a crucial component of desmosomes and also is important in signalling, metabolic and transcriptional activities.

Objectives – The main objective was to evaluate the role of PKP2 in CAD by investigating PKP2 expression and desmosome structure in nonlesional skin from CAD-affected (carrying the top GWAS SNP risk allele) and healthy GSDs. We also aimed at defining the cell types in the skin that express PKP2 and its intracellular location.

Animals/Methods – Skin biopsies were collected from nine CAD-affected and five control GSDs. The biopsies were frozen for immunofluorescence and fixed for electron microscopy immunolabelling and morphology.

Results – We observed the novel finding of PKP2 expression in dendritic cells and T cells in dog skin. Moreover, we detected that PKP2 was more evenly expressed within keratinocytes compared to its desmosomal binding-partner plakoglobin. PKP2 protein was located in the nucleus and on keratin filaments attached to desmosomes. No difference in PKP2 abundance between CAD cases and controls was observed.

Conclusion – Plakophilin-2 protein in dog skin is expressed in both epithelial and immune cells; based on its sub-cellular location its functional role is implicated in both nuclear and structural processes.

Introduction

In a genome-wide association study (GWAS) we identified a locus on canine chromosome 27 (CFA 27) associated with canine atopic dermatitis (CAD) in German shepherd dogs (GSDs). The strongest genetic association was detected near the PKP2 gene encoding the protein plakophilin-2. Plakophilin proteins are crucial for the proper assembly of desmosomes, which are intercellular mechanical junctions that contribute to strength and integrity in tissues, such as the myocardium and the epidermis, that exhibit mechanical stress. Plakophils, which belong to the armadillo protein family, bind to plakoglobin and are essential for recruiting desmoplakins to the desmosomal plaque, thereby providing an important linkage to the stabilizing and stress-bearing intermediate filaments inside the cell. Plakophilins are also known to be involved in multiple signalling and metabolic processes, and in transcriptional activities. Fine-mapping of the risk locus in GSDs identified genetic variants located in tissue-specific enhancers and suggested the possibility of alterations in PKP2 gene expression in CAD-affected GSDs. This suggests that PKP2 expression may be altered in the skin of GSDs carrying the risk variants compared to dogs without the risk variants. Expression patterns of the adhesion molecules cornodesmosin, desmoglein-1, desmocollin-1, claudin-1 and E-cadherin were compared in dog skin with acute CAD lesions and healthy skin from the same...
dogs, and the most striking differences were found for corneodesmosin and claudin-1. This implies that differential expression of desmosomal proteins is involved in the pathogenesis of atopic dermatitis (AD) in dogs and that PKP2 could also contribute to the pathogenesis of CAD.

In this present study, we collected skin biopsies from nine GSDs with CAD, either homozygous or heterozygous for the risk allele at the top GWAS-single nucleotide polymorphism (SNP), and five healthy control GSDs homozygous for the control allele. These samples were submitted to studies using immunofluorescence and electron microscopy (EM). We aimed at investigating the role of PKP2 in dog skin and evaluated the overall PKP2 expression in dog skin by defining which cell types and where in the cell PKP2 is localized. We also examined dog skin morphology with the focus on desmosomes and searched for potential differences between CAD cases and GSD controls in terms of the intensity of PKP2 expression and desmosome structure, with regards to previous GWAS results.

**Materials and methods**

**Sampling and ethics statement**

Skin biopsies (6 mm in diameter) were collected from 14 GSDs: nine diagnosed with CAD and five healthy controls (see the inclusion criteria below and Table 1) and also from one greyhound (unaffected by CAD and euthanized due to unrelated reasons). The GSDs were part of a previous study of CAD. The genotype at the top-associated GWAS SNP defined the dogs as either homozygous risk, heterozygous risk or homozygous control. From each dog we collected three biopsies of skin from the dorsal trunk and three skin biopsies from the axilla area. The biopsies were collected from nonlesional axilla and dorsal skin to represent typical atopy affected and nonaffected skin locations, respectively. The CAD cases were under, or had recently been receiving, treatment and the skin was therefore defined as nonlesional. Owner consent was sought and the study was approved by the Swedish Animal Ethical Committee (no. C138/12) and the Swedish Animal Welfare Agency (no. 31-1711/10).

**CAD phenotype characterization**

The CAD cases were between 6 and 11 years old at the time of sampling and fulfilled established criteria, with clinical signs compatible with CAD, neither prior to nor at the time of sampling. The information was based on owner questionnaire and clinical examination.

**Skin biopsy samples**

Two of the skin biopsies were split in half by a cut perpendicular to the skin surface. Three pieces per location were used in this study and were fixed as follows: (i) embedded in OCT cryomount (Histolab; Gothenburg, Sweden) in a plastic mould and frozen in an isopentane bath, cooled with dry ice, (ii) fixed in 4% paraformaldehyde with 0.2% glutaraldehyde for EM-immunolabelling, and (iii) fixed in 2.5% glutaraldehyde for EM. The fourth piece, fixed in 4% paraformaldehyde and embedded in paraffin, and the third whole biopsy, treated with RNA-later, were not included in this study.

**Immunofluorescence microscopy**

Five or 30 μm thick sections were cut from frozen skin blocks and mounted on Super frost plus glass slides (Gerhard Menzel GmbH; Braunschweig, Germany). Immunofluorescence for 5 μm sections was carried out as follows: the slides were thawed at room temperature (RT) for 30 minutes, fixed in pre-cooled acetone for 5 minutes at 4°C and air-dried at RT for 30 minutes. The sections were washed three times in phosphate buffered saline (PBS, pH 7.4) and then blocked in Background sniper blocking solution (Biocare Medical; Concord, CA, USA) for 10–15 minutes at RT. For double staining, a mix of two primary antibodies diluted in PBS with 1% bovine serum albumin (BSA, Sigma-Aldrich; St Louis, MO, USA) was added to the sections and incubated at 4°C overnight. Antibodies and specific dilutions are reported in Table S1, briefly slides were stained for CD3, CD4, CD11c, Junction plakoglobin (JUP), MHC-II and Plakophilin-2. Slides were washed three times with PBS. A mix of two fluorescently labelled secondary antibodies (Table S2) specific for the primary antibodies was diluted in PBS with 1% BSA added and incubated for 30 minutes at RT. The slides were washed three times in PBS. The sections were then stained with DAPI (4’,6-diamidino-2-phenylindole) for 5 minutes followed by washing four times in PBS. The sections were mounted in Fluromount G anti-fade medium (Southern Biotech; Birmingham, AL, USA) and covered with a cover glass fastened with nail polish and stored at 4°C until analysed by using a LSM 700 Confocal Laser Scanning microscope (Zeiss; Oberkochen, Germany) and LSM software Zen black (Zeiss).

**Table 1. Details of the dogs included in the study of PKP2 expression in the skin**

| Dog ID | Sex   | CAD     | Genotype top GWAS SNP | Represented in figure |
|--------|-------|---------|-----------------------|----------------------|
| 1      | Female| Case    | Homozygote risk       | 1b–d, S1a, S1c, S3b–d |
| 2      | Male  | Case    | Homozygote risk       |                      |
| 3      | Male  | Case    | Homozygote risk       |                      |
| 4      | Female| Case    | Heterozygote risk     | S1b, S1d             |
| 5      | Female| Case    | Heterozygote risk     |                      |
| 6      | Male  | Case    | Heterozygote risk     |                      |
| 7      | Female| Case    | Heterozygote risk     |                      |
| 8      | Male  | Case    | Heterozygote risk     | 1a                   |
| 9      | Male  | Case    | Heterozygote risk     |                      |
| 10     | Male  | Control | Homozygote control    | 2b                   |
| 11     | Female| Control | Homozygote control    | 3a, 3b, S6a, S6b     |
| 12     | Male  | Control | Homozygote control    | S2                   |
| 13     | Male  | Control | Homozygote control    | 2a, S3a, S4a–c       |
| 14     | Female| Control | Homozygote control    |                      |

CAD canine atopic dermatitis, GWAS genome-wide association study, SNP single nucleotide polymorphism.
For triple staining, four additional steps were added to the protocol above. After the step where secondary antibodies had been washed off, a third primary antibody was added diluted in PBS with 1% BSA and incubated for 1 h at RT or at 4°C overnight. The slides were washed three times in PBS. Fluorescence labelled secondary antibody, specific for the primary antibody, diluted in PBS with 1% BSA, was incubated for 30 minutes at RT and the slides were washed three times in PBS.

For the staining of 30 μm sections, slight modifications of the triple-staining protocol above were applied. The acetone fixation step was increased to 7 minutes. After the initial wash, a permeabilization step was added where the sections were incubated for 10 minutes in PBS with 0.2% Triton X-100 (Sigma-Aldrich) at RT followed by washing three times in PBS. All subsequent washing steps were increased to four washes and the two last washes lasted for 10 minutes. The primary antibody incubations were performed overnight at 4°C or for 3–4 h at RT. All secondary antibodies were incubated for 2 h at RT. The final wash in PBS after the DAPI staining was performed five times.

**Evaluation of the intensity of PKP2 expression in dendritic cells using immunofluorescence**

The intensity of PKP2 protein expression in dendritic cells was compared between cases homozygous for the top GWAS SNP 27:19,140,837 (n = 5), heterozygote cases (n = 6) and controls (n = 6) for the control allele at the top GWAS SNP (n = 5) in skin triple-stained with antibodies specific for PKP2, MHC II and CD11c. Dendritic cells were defined as cells with clear dendritic cell shape (i.e. presence of dendrites) that also expressed CD11c and MHC II, or cells with moderate to high expression of CD11c and MHC II. For each sample, four z-stacks of 92.4 × 92.4 μm spanning a 30 μm section were analysed and the 10 dendritic cells with the strongest PKP2 staining were chosen for further measurements. Using Fiji software, each cell was outlined by a polygon selection encompassing only this cell and using a triangle-threshold within this selection, the area of the PKP2 expression could be defined and the intensity of PKP2 expression measured in each 0.6 μm slice. PKP2 protein is expressed throughout the cell; hence the area of PKP2 expression equals the area of the cell. The intensity mean per slice × area of the cell was calculated for each slice and the total intensity of all slices divided by volume of the cell (area of each slice × 0.6 μm) gave a measurement defined as intensity/μm.3

**Immunoelectron microscopy for PKP2**

Dog skin samples fixed in 4% paraformaldehyde with 0.2% glutaraldehyde were embedded in Lowicryl® K4M (Polysciences; Warrington, PA, USA). Immunolabelling was performed as follows: ultra-thin, 50 nm sections were blocked in 1% BSA (Sigma-Aldrich) in PBS for 20 minutes, incubated with primary antibody against PKP2, as shown in the Western blot method given below (Sigma-Aldrich) in 1:10 dilution in TBS (Tris-buffered saline) pH 7.4 overnight at 20°C. The sections were washed four times in TBS pH 7.4 and incubated with goat anti-rabbit antibody conjugated with 20 nm gold particles (115-205-144, Jackson Immunoresearch; West Grove, PA, USA) diluted 1:100 in TBS pH 8.3 for 2 h. Next, the sections were washed four times in TBS pH 7.4 and rapidly rinsed in distilled water (dH2O). Thereafter, they were incubated in 5% uranyl acetate (Agar Scientific Ltd; Stansted, UK) and propylene oxide (1:1) for 1 h, followed by two changes of 100% resin, the first for 2–4 h and the last overnight. Subsequently, samples were embedded in capsules in newly prepared agar resin and left for 1 h and then polymerized at 60°C for 48 h. Ultrathin sections (50–60 nm) were contrasted in 5% uranyl acetate and lead citrate for 10 and 2 minutes, respectively. Sections were examined and figures were acquired as described in the immun-electro microscope section.

Skin samples from the axilla region of CAD affected GSDs, heterozygote (n = 6) and homozygote (n = 3) for the risk allele at the GWAS SNP 27:19,140,837, were compared with samples from five healthy GSDs homozygous for the control allele. The length of all desmosomes in three keratinocytes per sample, at similar locations per tissue section of 20 cells, were examined at 80 kV in a Tecnai G2 transmission electron microscope.

Western blot analysis

For protein extraction, canine skin from the axilla region from the greyhound and canine left ventricle cardiac muscle from a great dane dog (supplied by Ingrid Ljungvall and colleagues) were used. Frozen OCT cryomount was removed from two skin tissue specimens by dissolving the OCT cryomount in PBS pH 7.2. Skin tissues were pooled before homogenization. Homogenization was performed using Precellys Evolution (Bertin Technologies; Montigny le Bretonneux, France) with Precellys CK14 2 mL tubes. Tissues were homogenized 2 × 30 s at 6,500 rpm in a shaker (Precellys 24, Bertin Corporation, MD, USA) in 0.5 mL (cardiac muscle) or 0.3 mL (skin) PBS pH 7.2 containing 2% n-Octylglucoside (Santa Cruz Biotechnology Inc.; Dallas, TX, USA) and 1% Triton X-100 (Sigma-Aldrich) with one Pierce protease and phosphatase inhibitor mini tablet (Thermo Scientific; Rockford, IL, USA) dissolved in 10 mL of buffer immediately before use. Homogenates were centrifuged at 17,000 × g for 30 minutes at 4°C and supernatant protein concentration was determined using Bradford method (Bio-Rad Laboratories AB; Solna, Sweden) with BSA (Merck KGaA, Darmstadt, Germany) as standard. Homogenates were stored at −20°C until analysis.

Protein extracts from canine skin and heart (left ventricle) tissues (100 μg protein) were separated on 12% SDS-polyacrylamide gels (ClearPAGE precast gels, C.B.S. Scientific Company Inc.; Del Mar, CA, USA) and transferred to polyvinylidene difluoride membranes (Immobilon-FL, Millipore Corporation; Bellerica, MA, USA) at 0.24 amperie for 1 h using protein transfer system (Bio-Rad Laboratories AB). The membranes were blocked in TBST (PBS with 0.1% Tween 20) plus 5% nonfat milk powder for 1 h at RT and then incubated with rabbit anti-human PKP2 antibody (Sigma-Aldrich; Stockholm, Sweden) or mouse anti-human JUP (junctional plakoglobin) antibody (Sigma Aldrich) at 1:200 dilution in TBST plus 5% nonfat milk powder at 4°C O/N. After washing three times with TBST the membranes were then incubated with anti-rabbit respective anti-mouse IgG, hors eradish peroxidase linked secondary antibodies (GE Healthcare;
Little Chalfont Buckinghamshire, UK) at 1:5,000 dilution in TBST plus 5% nonfat milk powder for 2 h at RT, washed three times in TBST and detected by the enhanced chemiluminescence immunodetection system (ECL prime western blotting detection reagent, GE Healthcare). The images were captured by using Chemidoc touch imaging system (Bio-Rad Laboratories AB).

Statistical analysis
The Mann-Whitney test was used for statistical analysis. When comparing multiple groups the One-way ANOVA Kruskal-Wallis test with Dunn’s post hoc test was used. *P*-values lower than 0.05 were considered significant. All analyses were performed using Graphpad Prism™ 4.0 software (GraphPad Software Inc.; La Jolla, CA, USA).

Results
Immunofluorescence to study PKP2 expression in dog skin
In order to detect and evaluate PKP2 protein expression in dog skin, the sample sections were stained with an anti-PKP2 antibody. We initially evaluated dorsal and axilla skin from one control dog (greyhound) for optimization of permeabilization, incubation times and dilutions of all antibodies (including double and triple staining). We detected PKP2 expression in keratinocytes as well as T cells and dendritic cells and concluded that no differences could be detected between axilla and dorsal skin. Because skin from the axilla represents the part of the dog’s body typically affected by lesions in CAD it was regarded as the most relevant tissue for detection of potential differences between CAD-case and control dog skin. The following studies were therefore carried out in axilla skin from the 14 sampled GSDs.

Plakophilin-2 was expressed in keratinocytes in both CAD dogs and control GSDs. In some of the keratinocytes, the PKP2 staining was somewhat stronger close to the plasma membrane but we did not observe an expression gradient throughout the keratinocyte layers (Figure S1a). The observed staining was specific for the keratinocytes as shown by the lack of staining in the negative control. There was nonspecific staining of the stratum corneum caused by the secondary antibody, but this was clearly not affecting the staining in the keratinocytes or the other cells in the skin (Figure S1b). At a higher magnification an increase in expression of PKP2 closer to the plasma membrane was more evident. However, the difference in strength compared to the rest of the cytoplasm was small (Figure S1c). Next, the location of PKP2 proteins was compared to the location of plakoglobin in axilla skin. Plakoglobin is one of PKP2’s binding partners within the desmosomes. The double staining with anti-PKP2 and anti-plakoglobin antibodies showed that plakoglobin staining was restricted to the keratinocytes in the epidermis, whereas PKP2 staining was seen also in other cells of the epidermis and dermis (Figure 1a). Within the keratinocytes in the epidermis, PKP2 expression was more homogenous in the cytoplasm compared to plakoglobin, and the location near the plasma membrane was more evident and distinct for plakoglobin compared to PKP2 (Figure 1b-d). The same pattern was seen in skin samples from all dogs (n = 14).

We detected PKP2 expression also in other cells in the axilla skin. Some of these cells displayed even higher expression intensity than keratinocytes, including cells that appeared to be dendritic cells or melanocytes, and endothelial cells judging by the cell shapes and locations (Figure S2), as well as cells that could possibly be T cells due to their location close to vessels. To further examine some of these cell types (other than keratinocytes) that expressed PKP2, we performed triple staining with antibodies against the T-cell markers CD4 and CD3, and the dendritic cell markers CD11c and MHC II. Both T cells (Figure 2a and Figure S3) and dendritic cells (Figure 2b and Figure S4) showed strong PKP2 expression in both the cytoplasm and in the nuclei. We did not note any PKP2-positive cells with dendrites in the epidermis that were not positive for CD11c/MHC II; however, we cannot rule out possible PKP2 expression in melanocytes because we did not use any melanocyte-specific makers.

Intensity of PKP2 expression in skin dendritic cells
In order to further investigate the expression of PKP2 in dendritic cells and to compare the expression pattern between CAD cases and controls, we calculated the intensity of PKP2 expression in the ten dendritic cells with the highest intensity per skin sample. This was performed on axilla skin samples from all 14 dogs. The cells were chosen from four z-stacks triple stained for PKP2, CD11c and MHC II. The average intensity per μm² for the different groups were as follows: homozygous cases 16.3 (ranging from 10.4 to 26.5), heterozygous cases 18.9 (12.7–23.7) and controls 14.8 (11.3–20.5; Figure S5). No significant differences between group averages were detected using Mann-Whitney t-test (two tailed with 95% confidence intervals), for cases versus controls (P= 0.44), for homozygous cases versus controls (P= 0.79) or for heterozygous cases versus controls (P= 0.18).

PKP2 expression in dog skin using immuno-electron microscopy
In order to further investigate the expression of PKP2 in epidermal tissue from dog skin. For this purpose, ultrathin sections from one healthy control GSD were immunolabelled with anti-PKP2 antibodies and analysed using immuno-electron microscopy. Gold particles, indicating binding of anti-PKP2 antibodies, were detected in the vicinity of desmosomes on keratin filaments attached to desmosomes (Figure 3a) and in the majority of the nuclei (Figure 3b). The negative control, with omission of the primary antibody, only showed very few gold particles in the section. A few of these gold particles were located in these nuclei (Figure S6a) but all other nuclei were negative for staining. No gold particles were located in or near the desmosomes in the negative control (Figure S6b).

Skin morphology – electron microscopy
In order to detect possible differences in skin morphology between cases and controls, we evaluated the skin samples using TEM. Considering desmosome length and number of desmosomes in relation to cell membrane length, we could not detect any significant differences between the groups. To assess if a defect in binding of keratin filaments to the desmosomes could result in an increased risk for developing CAD, we also analysed the levels of visible keratin filaments in the cytoplasm and the
number of desmosomes where the binding of keratin filaments appeared to be uneven or unstructured. In this analysis, we found no significant differences between CAD cases (neither separated into either heterozygote or homozygote cases or considered as one case group) and controls using Kruskal–Wallis one-way ANOVA (Figure S7a–g).

Western blot analysis
Because the anti-PKP2 and anti-JUP antibodies were developed for human proteins, we confirmed specific binding to canine PKP2 and JUP by performing Western blot analyses using extracts from canine skin and heart tissues (left ventricle). The results for anti-PKP2 and anti-JUP antibodies showed specific binding to target proteins of the expected sizes (PKP2 93 kDa and JUP 82 kDa) for both these two canine proteins thus validating the across-species specificity of both antibodies (Figure S8).

Discussion
The use of EM immunolabelling of ultrathin skin sections confirmed PKP2 located in the nuclei and on keratin filaments in close vicinity to desmosomes. Previously, EM

© 2017 The Authors. Veterinary Dermatology published by John Wiley & Sons Ltd on behalf of the ESVD and ACVD, 28, 377–e88.
analyses of human skin using immunolabelling with anti-PKP2 antibodies, showed binding near the cytoplasmic side of the desmosomal plaque in stratified epithelial tissue. This suggests that the subcellular expression of PKP2 in keratinocytes is similar in both human and dog skin. Previous studies in dogs have shown that there is a gradient in expression of PKP1, with stronger expression in the upper layers of the skin compared to the layers closer to dermis. This is in concordance with the expression in human skin. The same human study also showed that PKP2 expression was higher in the lower layers and weaker in the top layers of human skin whereas PKP3
was expressed more evenly in all skin layers.12 We could not observe any gradient in expression of PKP2 in the studied dog skin samples using IF. However, the epidermis of the axilla skin was very thin, only 1–3 keratinocytes thick. The expression pattern of PKP2 differed from its binding-partner plakoglobin, which was more restricted to cell membranes. This indicates a role for plakoglobin more limited to desmosomes in contrast to the multiple functions implicated for PKP2.

We confirmed that CD11c+ and MHC II+ cells (i.e. dendritic cells) as well as CD4+ and CD3+ cells (i.e. T cells) in dog skin express PKP2. Expression of PKP2 in T cells and dendritic cells in skin is a novel finding. Although, according to BioGPS (www.biogps.org) mRNA expression of PKP2 has been detected in human T cells and CD3+ myeloid cells in the blood (e.g. myeloid dendritic cells) but not in epidermal dendritic cells.13 Interestingly, in skin samples from the Human Protein Atlas (www.proteinatlas.org), anti-PKP2 antibodies showed reactivity to several non-keratinocyte cells that stained with a higher intensity than the keratinocytes in the samples presented. This is very similar to the staining pattern that we observed in dog skin. On the Protein Atlas website, there is no report of observed staining with the anti-PKP2 antibody in Langerhans cells (LCs). However, we studied the CD11c+ (ITGAX)-staining from the Protein Atlas site and detected positive staining of cells looking very similar to the strongest PKP2-stained cells (i.e. defined here as dendritic cells) in the skin samples from our study. This suggests that PKP2 is expressed in dendritic cells also in human skin. LCs are specialized dendritic cells in the skin that are activated by perturbation of the stratum corneum. The activated LCs extend their dendrites through the tight junction barrier located in the stratum spinosum of the skin and thereby efficiently capture allergens, which they present on their MHC molecules to the T cells.14 This initiates an immune response that can result in AD. Thus, the strong expression by the CAD-candidate gene PKP2 by both T cells and LCs may be of high relevance for CAD development.

We did not detect any significant differences in PKP2 protein expression between the CAD cases and controls used in our study. This is in concordance with mRNA sequence data from axilla skin biopsies from nine of the dogs used in the present study. In that data there were no PKP2 expression differences between CAD cases and controls. Instead, preliminary analyses of mRNA expression data suggest differences related to treatment effects (Tengvall et al., unpublished data). Thus, possibly PKP2 gene and protein expression differences between CAD cases and controls may be masked by treatment, or relate to other functions by this protein, potentially even in other tissues. It may also be due to other genes being affected by the genetic risk variants. Nevertheless, both mRNA and protein expression data are of strong value to further evaluate the potential role of PKP2 in the pathogenesis of CAD. The reason for not detecting any differences may be due to the lack of untreated lesional skin from CAD cases. All the CAD dogs were in remission by collection from nonlesional skin. Protein expression in the nuclei of the keratinocytes.18 We confirmed PKP2 expression in the nuclei of keratinocytes, dendritic cells and T cells. However, with the current methods used, we could not evaluate the functional role of PKP2 in the nuclei or compare CAD cases with controls regarding PKP2 expression in the nuclei.

We were unable to define any differences between CAD cases and controls with respect to PKP2 protein expression pattern and intensity nor desmosomal morphology. Perhaps, an altered PKP2 expression associated with the defined genetic variants affects other pathways,
mechanisms and/or cell types, undetectable by the currently used materials and methods. The strong expression of PKP2 in LCs and T cells may imply that an altered expression of PKP2 in these cell types may correlate to atopic disease. Or possibly, nuclear processes of stratified epithelial cells may be affected rather than structural properties of the basal epithelial layers. Moreover, intestine integrity and allergen uptake is of high relevance in atopic skin manifestations\textsuperscript{20,21} and a rather high mRNA and protein expression of PKP2 has been detected in the digestive tract (www.proteinatlas.org). Thus, this may be a target tissue for this risk locus in CAD development as GSDs are known to be particularly prone to gastrointestinal- nal problems and are also affected by cutaneous adverse food reactions. An altered regulation of PKP2 mRNA expression in the intestinal epithelial cells may lead to changes in PKP2 protein levels that potentially could disturb desmosome stability and subsequently increase allergen penetration and uptake through the intestine.

In conclusion, PKP2 expression was detected in keratinocytes, dendritic cells and T cells in the epidermis and dermis, and was more evenly expressed within keratinocytes compared to its desmosomal binding-partner plakoglobin. The expression of PKP2 in T cells and dendritic cells in skin is likely to be of relevance for CAD development and has previously not been reported. PKP2 protein was located in the nucleus as well as on keratin filaments attached to desmosomes. No difference in PKP2 abundance between CAD cases and controls was observed. We report a wide expression pattern of PKP2 in dog skin that is in accordance with previous reports suggesting that this protein contributes to multiple functions within the cell including both structural and transcriptional activities.

Acknowledgements

We would like to thank all owners of the dogs that participated in this study as well as the veterinarians Rebecka Frey, Carina Kubacki, Ylva Sjöström and Susanne Åhman who helped with the collection of biopsies at different veterinary clinics in Sweden. Help with immuno-electron microscopy including preparation of samples, preparation of samples for morphology evaluation and expert guidance in electron microscopy, as well as guidance and use of the confocal microscopy equipment, was provided by the staff at the BioVis facility at SciLifeLab, Uppsala University, Uppsala, Sweden. We thank Anna Svensson at the Department of Clinical Sciences, at the Swedish University of Agricultural Sciences (SLU, Uppsala, Sweden, for protein extraction service and Susanne Gustafsson at the Canine Biobank (SLU and Uppsala University) for sample management. We also would like to thank Ingrid Ljungvall, Jens Häggström and Asa Ohlsson Andersson for supplying us with canine heart tissue for the western blot analyses.

References

1. Tengvall K, Kierczak M, Bergvall K et al. Genome-wide analysis in German shepherd dogs reveals association of a locus on CFA 27 with atopic dermatitis. PLoS Genet 2013; 9: e1003475.
2. Chen X, Bonne S, Hatzfeld M et al. Protein binding and functional characterization of plakophilin 2. Evidence for its diverse roles in desmosomes and beta-catenin signaling. J Biol Chem 2002; 277:10,512–10,522.
3. Rasmussen TB, Nissen PH, Palmefeldt J et al. Truncating plakophilin-2 mutations in arthrogryphic cardiomyopathy are associated with protein haploinsufficiency in both myocardium and epidermis. Circ Cardiovasc Genet 2014; 7: 230–240.
4. Green KJ, Simpson CL. Desmosomes: new perspectives on a classic. J Invest Dermatol 2007; 127: 2,499–2,515.
5. Bass-Zubek AE, Godsel LM, Delmar M et al. Plakophilins: multi-functional scaffolds for adhesion and signaling. Curr Opin Cell Biol 2009; 21: 708–716.
6. Tengvall K, Kozyrev S, Kierczak M et al. Multiple regulatory variants located in cell type-specific enhancers within the PKP2 locus form major risk and protective haplotypes for canine atopic dermatitis in German shepherd dogs. BMC Genet 2016; 17: 97.
7. Olivry T, Dunston SM. Expression patterns of superficial epidermal adhesion molecules in an experimental dog model of acute atopic dermatitis skin lesions. Vet Dermatol 2015; 26: 53–56, e17–18.
8. Favrot C, Steffan J, Seewald W et al. A prospective study on the clinical features of chronic canine atopic dermatitis and its diagnosis. Vet Dermatol 2010; 21: 23–31.
9. Schindelin J, Arganda-Carreras I, Frise E et al. Fiji: an open-source platform for biological-image analysis. Nat Methods 2012; 9: 676–682.
10. Mertens C, Kuhn C, Franke WW. Plakophilins 2a and 2b: constitutive proteins of dual location in the karyoplasm and the desmosomal plaque. J Cell Biol 1996; 135: 1,009–1,025.
11. Bzikova P, Linder KE, Olivry T. Immunomapping of desmosomal and nondesmosomal adhesion molecules in healthy canine foot-pad, haired skin and buccal mucosal epithelia: comparison with canine periphilic foliaiceous serum immunoglobulin G staining patterns. Vet Dermatol 2011; 22: 132–142.
12. Desai BV, Harmon RM, Green KJ. Desmosomes at a glance. J Cell Sci 2009; 122: 4,401–4,407.
13. Su AI, Wiltshire T, Batalov S et al. A gene atlas of the mouse and human protein-encoding transcripts. Proc Natl Acad Sci USA 2004; 101: 6,062–6,067.
14. Villadangos JA, Heath WR. Life cycle, migration and antigen presenting functions of spleen and lymph node dendritic cells: implications of the Langerhans cells paradigm. Semin Immunol 2005; 17: 262–272.
15. Wood SH, Clements DN, Ollier WE et al. Gene expression in canine atopic dermatitis and correlation with clinical severity scores. J Dermatol Sci 2009; 55: 27–33.
16. Joshi-Mukherjee R, Coombs W, Musa H et al. Characterization of the molecular phenotype of two arrhythmogenic right ventricular cardiomyopathy (ARVC)-related plakophilin-2 (PKP2) mutations. Heart Rhythm 2008; 5: 1,715–1,723.
17. Grossmann KS, Grund C, Huelskens J et al. Requirement of plakophilin 2 for heart morphogenesis and cardiac junction formation. J Cell Biol 2004; 167: 149–160.
18. Heuer S, Muhmer M, Wratten D et al. The desmosomal plaque proteins of the plakophilin family. Dermatol Res Pract 2010; 2010: 101452.
19. McMillan JR, Haftek M, Akiyama M et al. Alterations in desmosome size and number coincide with the loss of keratinocyte cohesion in skin with homozygous and heterozygous defects in the desmosomal protein plakophilin 1. J Invest Dermatol 2003; 121: 96–103.
20. Marsella R, Samuelson D. Unravelling the skin barrier: a new paradigm for atopic dermatitis and house dust mites. Vet Dermatol 2009; 20: 533–540.
21. Marsella R, Samuelson D, Doer K. Transmission electron microscopy studies in an experimental model of canine atopic dermatitis. Vet Dermatol 2010; 21: 81–88.
Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1. Primary antibodies used for indirect immunofluorescence.

Table S2. Secondary antibodies for immunofluorescence.

Figure S1. PKP2 expression in keratinocytes.

Figure S2. PKP2 expression in dendritic cells and endothelial cells in dog skin.

Figure S3. PKP2 expression in T cells.

Figure S4. PKP2 expression in dendritic cells.

Figure S5. Intensity of PKP2 expression in dendritic cells.

Figure S6. Negative controls for electron microscopy immunohistochemistry.

Figure S7. Assessment of desmosomes and keratin filaments in keratinocytes.

Figure S8. Western blot confirming antibody specificity to canine proteins.

Résumé
Contexte – La dermatite atopique canine (CAD) est une dermatose allergique prurigineuse et inflammatoire due à des interactions entre les facteurs génétiques et environnementaux. Précédemment, un locus de risque significatif sur le chromosome 27 de chien du génome sauvage du Berger Allemand (GSD) a été identifié et la Plakophilin-2 (PKP2) a été défini comme principal gène candidat. PKP2 constitue un composant crucial des desmosomes et est également important dans les activités signal, métabolique et transcription.

Objectifs – L’objectif principal était d’évaluer le rôle de PKP2 dans la CAD par étude de l’expression de PKP2 et de la structure desmosomique dans la peau non lésionnelle de GSDs atteint de CAD (porteurs de l’allèle GWAS SNP majeur de risque) et des GSD sains. Nous voulons aussi définir le type cellulaire qui exprime PKP2 et sa localisation intracellulaire.

Sujets/Méthodes – Des biopsies cutanées ont été prélevées pour neuf GSDs atteints de CAD et cinq contrôles sains. Les biopsies ont été congelées pour immunofluorescence et fixées pour morphologie et immunomarquage par microscopie électronique.

Résultats – Nous observons l’expression de PKP2 dans les cellules dendritiques des cellules T dans la peau de chien. En outre, nous détectons que PKP2 a été plus intensément exprimé par les kératinocytes comparé à la plakoglobine. La protéine PKP2 a été localisée dans le noyau et sur des filaments de kératine attachés aux desmosomes. Aucune différence de quantité de PKP2 entre les cas atopiques et contrôles n’a été observée.

Conclusion – La protéine Plakophiline-2 dans la peau de chien est exprimée à la fois par les cellules épithéliales et par les cellules immunitaires et, basée sur sa localisation subcellulaire, son rôle fonctionnel est impliqué à la fois dans les processus nucléaires et structurels.

Resumen
Introducción – La dermatitis atópica canina (CAD) es una enfermedad inflamatoria y pruriginosa alérgica de la piel causada por interacciones entre factores genéticos y ambientales. Anteriormente, se identificó un locus de riesgo significativo en el genoma del cromosoma 27 canino para CAD en perros de Pastor Alemán (GSD) y placofilina-2 (PKP2) se definó como el gen candidato más posible. PKP2 constituye un componente crucial de los desmosomas y también es importante actividades de señal, metabólicas y transcripcionales.

Objetivos – El objetivo principal fue evaluar el papel de la PKP2 en CAD mediante la investigación de la expresión de PKP2 y la estructura desmosomica en la piel no afectada de perros de raza GSD con CAD (portadores del alelo GWAS SNP de alto riesgo) y sanos. También se intentó definir los tipos de células en la piel que expresan PKP2 y su ubicación intracelular.

Animales/Métodos – Se recolectaron biopsias cutáneas de nueve perros afectados por CAD y cinco controles de raza GSD. Las biopsias se congelaron para realizar inmunofluorescencia y se fijaron para inmunomarqueo y estudio morfológico mediante microscopia electrónica.

Resultados – Se observó por vez primera la expresión de PKP2 en células dendríticas y células T en la piel del perro. Por otra parte, detectamos que PKP2 fue más uniformemente expresada dentro de queratinocitos en comparación con su proteína de unión en desmosomas, plakoglobina. La proteína PKP2 se localizó en el núcleo y en filamentos de queratina unidos a desmosomas. No se observó diferencia en la abundancia de PKP2 entre los casos afectados por CAD y los controles.

Conclusión – La proteína placofilina-2 en la piel de perro se expresa tanto en las células epiteliales como inmunitarias, y basados en su ubicación subcelular su papel funcional estaría implicado en procesos nucleares y estructurales.

Zusammenfassung
Hintergrund – Die atopische Dermatitis des Hundes (CAD) ist eine entzündliche und juckende allergische Hauterkranzung, die durch Wechselwirkungen von genetischen und Umweltfaktoren verursacht wird. Bis her wurde ein Genom-weiter signifikanter Risikolokus am caninen Chromosom 27 bei Deutschen
Schäferhunden (GSSs) für CAD identifiziert und Plakophilin-2 (PKP2) wurde als Top Kandidatengen definiert. PKP2 stellt eine wesentliche Komponente der Desmosomen dar und ist auch wichtig bei der Nachrichtenübermittlung, dem Stoffwechsel und bei der Transkription.

Ziele – Das Hauptziel war die Evaluierung der Rolle von PKP2 bei der CAD mittels Untersuchung der PKP2 Exprimierung und der Desmosomenstruktur in nichtveränderter Haut von Schäferhunden mit CAD (Träger des Top GWAS SNP Risikoalleles) und gesunden Schäferhunden. Wir versuchten auch die Zelltypen in der Haut, die PKP2 exprimieren zu definieren und ihre intrazelluläre Lokalisation zu bestimmen.

Tiere/Methoden – Es wurden Hautbiopsien von neun Schäferhunden mit CAD und fünf Schäferhunden als Kontrolle genommen. Die Biopsien wurden für eine Immunfluoreszenzuntersuchung eingefroren und fixiert für die Immunmarkierung und Morphologie-Analyse mittels Elektronenmikroskop.

Ergebnisse – Als neue Beobachtung fanden wir die Exprimierung von PKP2 in denritischen Zellen und in T Zellen der Hundehaut. Zusätzlich fanden wir, dass PKP2 im Vergleich zum desmosomalen Bindungspartner Plakoglobin innerhalb der Keratinozyten gleichmäßig exprimiert wurde. PKP2 Protein war im Kern lokalisiert und durch Keratinfilamente an den Desmosomen fixiert. Es wurde kein Unterschied im Ausmaß des fixierten PKP2 Protein innerhalb der Keratinozyten festgestellt, sondern es wurde ein einheitlicher Wandaufbau beobachtet.

Schlussfolgerung – Plakophilin-2 Protein in der Hundehaut wird sowohl in epithelialen wie auch in Immunzellen exprimiert und aufgrund seiner subzellulären Lokalisation wird seine funktionelle Rolle sowohl in Vorgängen im Kern wie auch in der aktiven Zelle vermutet.

要約

背景 – 犬アトピー性皮膚炎(CAD)は、遺伝的要因と環境的要因との相互作用によって引き起こされる炎症性および機能性アレルギー性皮膚疾患である。これまで、ジャーマンシェパードドッグ(GSD)のCADゲノムワイド解読により、その重要な遺伝子を含むためには、最も重要であると考えられるに至った。

目的 – 主要な目的は、CADに罹患しているGSD(GWASにおける最長SNP)陽性対象遺伝子を保有する非正常皮膚および健常GSDのPKP2発現およびデスモソーム構造を調べることにより、CADにおけるPKP2の役割を評価することであった。我々は、皮膚におけるPKP2の発現を発現する細胞の種類およびその細胞内の位置を同定することを目的とした。

考察

背景 – 犬アトピー性皮膚炎(CAD)は遺伝的関係性の相互作用で引き起こされる一種の炎症性アレルギー過敏性皮膚病である。有効な研究が、自身免疫性皮炎(GSD)の第20染色体上、存在する重要遺伝子であるクラ音器という非蛋白質性蛋白質PKP2(2PK)の発現が、最高リスクファクターであることを示した。

目的 – 主要な目的は、CADに罹患しているGSD(GWASにおける最長SNP)陽性対象遺伝子を保有する非正常皮膚および健常GSDのPKP2発現およびデスモソーム構造を調べることにより、CADにおけるPKP2の発現を発現する細胞の種類およびその細胞内の位置を同定することを目的とした。

動物/方法 – 9対のCADとGSDの皮膚組織を用いて皮膚細胞を採取。現実的な分類、免疫組織化学および組織学的観察を用いて検討した。

結果 – 我々は、その発現部位の特異性およびPKP2の発現を評価することを目的とした。

結論 – PKP2の発現部位の特異性およびPKP2の発現を評価することを目的とした。
Objetivos – O principal objetivo foi avaliar a função da PKP2 na DAC a partir da investigação na expressão de PKP2 e da estrutura dos desmossomos na pele alesional de cães com DAC (portadores do alelo de risco GWAS SNP) e PAs saudáveis.

Animais/métodos – Biópsias cutâneas foram coletadas de nove cães atópicos e cinco PAs saudáveis (controle). Os fragmentos foram congelados para imunofluorescência e fixados para imunomarcção e análise de morfologia em microscópio eletrônico.

Resultados – Observou-se a expressão de PKP2 em células dendríticas e células T em cães, um achado inédito. Além disto, detectou-se que PKP2 foi expressada mais uniformemente dentro de queratinócitos quando comparado com a placoglobina, seu complemento de ligação desmossômico. A proteína PKP2 foi localizada no núcleo e nos filamentos de queratina ligados aos desmossomos. Não foi observada diferença na abundância de PKP2 entre cães atópicos e cães controle.

Conclusão – A placofilina-2 é um proteína expressada tanto em células epiteliais quanto do sistema imunológico, e, baseado em sua localização subcelular, a sua função está relacionada tanto a processos nucleares quanto estruturais.