Epidermolysis bullosa simplex in sibling Eurasier dogs is caused by a PLEC non-sense variant

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Background – Plectin, a large linker protein found in many tissues, acts to connect components of the cytoskeleton to each other. In the epidermis, plectin binds keratin intermediate filaments to hemidesmosomes. A deficiency of plectin in the skin leads to blister formation in the basal layer and the disease epidermolysis bullosa simplex (EBS).

Hypothesis/Objectives – To describe a novel blistering disease that arose spontaneously in a litter of puppies.

Animals – Two female and one male 20-day-old Eurasier puppies, from a litter of six, were presented for evaluation of failure to thrive and then euthanized due to poor prognosis. The puppies had ulcers on the lips, tongue, nasal planum, paw pads and abdomen.

Results – Immunolabelling on frozen skin for basement membrane proteins revealed patchy and weak to absent staining for plectin as compared with strong linear staining in normal dogs. Ultrastructurally, hemidesmosomes were irregularly shaped and had loss of distinction between inner and outer plaques. Pedigree analysis supported an autosomal recessive mode of inheritance. A premature stop codon was discovered in exon 27 of PLEC that resulted in the production of a severely truncated protein.

Conclusion – The study describes the first documented spontaneous EBS associated with a PLEC variant in domestic animals.

Introduction

Epidermolysis bullosa (EB) is the name given to a group of blistering diseases that manifest as loss of epidermo-dermal integrity.1,2 The phenotypic changes are due to abnormalities in specific structural proteins within the epidermis and basement membrane zone. Characterization of EB is further subdivided into four major types based on the location of the subcellular defect. EB simplex (EBS) is the most superficial form and involves proteins in the cytoskeleton of basal or suprabasal keratinocytes. In junctional EB (JEB) blisters arise in the lamina lucida, whereas in dystrophic EB (DEB) the defect occurs in the superficial dermis at the level of anchoring fibrils. Kindler syndrome is a mixed pattern that has not been described in domestic animals.1,2 Light microscopy is generally unable to differentiate the subtypes of EB and some types of EB have undergone extensive reclassification.

The current approach to classification of EB in humans is an “onion skinning” method based on (i) subcellular location of the blister, (ii) clinical features, (iii) heritability and (iv) identification of the gene involved by immunohistochemical and mutational analysis.2 In EBS, the cleavage plane lies within the epidermis and may involve basal keratinocytes (basal EBS) or keratinocytes within the middle to upper layers of the epidermis (suprabasal EBS). Proteins involved in basal EBS include keratins 5 and 14, plectin, BPAG1e (BP230), exophilin 5 and kindling 1. Suprabasal EBS may involve transglutaminase 5 in the upper epidermis, or plakoglobin, plakophilin 1 and desmoplakin in the middle epidermis. The clinical subtypes relate to extent of lesions (e.g. generalized severe EBS versus localized EBS) or the presence of concurrent conditions (e.g. EBS with mottled pigmentation, EBS with muscular dystrophy).2,4-6

In humans, three subsets of plectin-associated EBS have been identified: EBS with muscular dystrophy (EBS-MD OMIM # 226670), EBS with pyloric atresia (EBS-PA OMIM #612138) and EBS-Ogna. OMIM #131950). EBS-MD and EBS-PA are autosomal recessive and have skin lesions with abnormalities in other organs. EBS-Ogna is autosomal dominant and characterized by relatively mild blistering without lesions in other organs.4-6 Plectin, which is encoded by PLEC, is a large protein found in many tissues (skin, bone, muscle and nervous
system); it links components of the cytoskeleton (e.g., actin microfilaments, microtubules, intermediate filaments) to the cell membrane.\(^5\),\(^7\),\(^8\) In basal keratinocytes, plectin and BPAG1e are the main components of the inner plaque of hemidesmosomes. Although all major forms of EB have been identified in domestic animals,\(^9\) this study documents the first spontaneous EBS associated with deleterious variant of plectin.

**Materials and methods**

Two female and one male 20-day-old Eurasier puppies, from a litter of six with an asymptomatic dam and sire, presented to the Matthew J. Ryan Veterinary Hospital of the University of Pennsylvania for failure to thrive. The puppies were underweight and approximately 50% smaller than the normal littermates. Physical examination revealed widespread ulcers on the lips, tongue, nasal planum, paw pads and abdomen with a positive Nikolsky sign (Figure 1). The puppies were diagnosed with probable JEB and were humanely euthanized due to poor prognosis. At the time of death, peripheral blood was obtained for DNA analysis and skin samples were fixed in modified Karnovsky’s fixative for ultrastructural analysis and snap frozen in liquid nitrogen for immunostaining and RNA analysis.

Skin samples for conventional transmission EM (TEM) were processed by standard techniques to evaluate the basal layer and basement membrane zone (BMZ).\(^1\)

Immunostaining for basement membrane proteins was performed to identify a defect in protein expression. Briefly, five micrometer frozen sections were cut from perilesional skin of the oral cavity (tongue or lip), pawpad and haired skin of the three EB-affected dogs. Sections were stained with a panel of antibodies specific for basement membrane proteins (BPAG1e, integrin alpha6 and beta4, collagen XVII, laminin 332 and collagen VII) as described previously\(^1\) with the addition of a rabbit polyclonal plectin antibody at dilution 1:100 (Table S1). All samples were stained for each individual protein on the same day to avoid any variability between assays. The pattern and intensity of staining were compared to those of normal controls.

Genomic DNA was extracted from EDTA blood obtained from all members of the family. Because there is no curated canine PLEC transcript or gene reference sequence available (RefSeq data from the National Center for Biotechnology Information, US National Library of Medicine, Bethesda, MD, USA), exons were determined by examination of the CanFam 3.1 reference genome assembly [NCBI assembly/GCF_000002285.3 (Broad CanFam3.1)] using the UCSC Genome Browser (http://genome.ucsc.edu/), including the Broad Institute CanFam3 Improved Annotation Data v1, which contains additional SNP and RNAseq data, including RNAseq from skin.

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**Figure 1.** Clinical images; Male and female 20-day-old Eurasier puppies with epidermolysis bullosa simplex. (a) Large ulcers on the paw pads, (b) inguinum and vulva, (c) prepuce and peripreputial skin, and (d) sloughing of the oral mucosa on the tongue.

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Exons and the intron–exon boundaries of the canine PLEC gene were sequenced. The human PLEC gene encodes several isoforms that differ primarily by the use of different first exons, which are followed by the same 31 additional exons to encode a protein of 4,684 amino acids for the most common isoform (RefSeq NM_201380). The canine orthologue of this transcript is encoded by 32 exons and codes for a protein of 4,686 amino acids that is 92.6% identical to the human protein. Primers (Table S2) for amplification of the canine exons were designed using commercially available software (DNAStar Inc.; Madison, WI, USA). Sequence data were analysed using Lasergene software (DNAStar Inc.) and the sequences compared to the respective canine PLEC region from the CanFam 3.1 reference sequence.

Results

Postmortem examination abnormalities were confined to the haired and nonhaired skin and oral cavity; significant lesions were not apparent in other organs including the gastrointestinal tract and skeletal muscle. Microscopic examination of lesions skin revealed clefts, vesicles and broad areas of epithelial detachment in both the haired skin and mucous membranes (lips, tongue, oral cavity, genitalia) with ulcers and fibrinosuppurative inflammation (Figure 2a). Periodic acid Schiff staining revealed weakly positive stain uptake on the floor of the blisters (Figure 2b). Transmission EM showed the lamina densa on the floor of the cleavage. Hemidesmosomes were located on the roof of the cleavage. The hemidesmosomes had an electron dense conical shape that attached to thickened keratin intermediate filaments. A loss of distinction was seen between inner and outer plaques (Figure 3).

Indirect immunofluorescence for basement membrane proteins revealed patchy and weak to absent plectin staining as compared with strong linear staining in normal dogs (Figure 4); other stains were unremarkable. A microscopic cleft was seen in biopsies from two of the three Eurasier puppies and, when present, cleavage was located above the stains for laminin 332 and collagen VII (not shown). These anomalies, which were restricted to sections stained for plectin, suggested that the PLEC gene encoding plectin might harbour a DNA variant leading to a defective expression of this protein.

A homozygous non-sense variant was identified in the affected puppies. The variant was located in exon 27 and predicted to truncate more than 70% of the open reading frame. A restriction fragment length polymorphism (RFLP) assay was developed to screen for the variant. DNA was amplified and digested with Bmt1 restriction enzyme (Table S3), which digests DNA when the disease-associated allele is present. The parents of the affected puppies were heterozygous for this non-sense variant and a clinically normal littermate was homozygous for the wild-type sequence (Figure 5a).

In order to verify that the variant found was not a polymorphism found in the general dog population, DNA was sampled from five different breeds (German shorthair pointer, American bulldog, Irish wolfhound, bull terrier and mixed breed; 25 dogs each). All 125 dogs were homozygous for the reference allele (G/G). Several silent and mis-sense variants in PLEC were also detected in DNA from the affected animal; many have been previously identified (Table S3).

Discussion

Plectin is an ubiquitous linker protein that serves to connect components of the cellular cytoskeleton to proteins in the skin, nervous system and skeletal muscle. The protein comprises four major domains: the amino-terminal or calponin homology domain, the plakin domain, the rod domain and the carboxy terminal domain. Plectin 1a is the only isoform known to bind to the beta unit of the integrin a6b4 via the plakin domain, adjacent to the amino terminus, and to keratin intermediate filaments via its carboxy terminal domain. Therefore, plectin serves as a vital structural component of the BMZ; as a bridge between the inner cytoskeleton to the basal lamina.

The importance of plectin in the BMZ was illustrated in the three Eurasier dogs in which a homozygous G to A variant in the PLEC gene resulted in the conversion of a tryptophan to a premature stop codon in exon 27. This resulted in a truncated 1,315 amino acid protein (normal 4,686 amino acids) and thus the loss of a large portion of

Figure 2. Photomicrographs of haired skin from Eurasier dog with epidermolysis bullosa simplex. (a) Note the large subepidermal vesicle. Haematoxylin and eosin 4x. (b) Weakly positive periodic acid Schiff staining (arrow) on the floor of the blister. 10x.
the rod domain and, more importantly, the C terminus that would bind to the keratin intermediate filaments. In this family of dogs, review of the pedigree, together with the mutational analysis, confirmed an autosomal recessive mode of inheritance.

Autosomal recessive, basal forms of EBS in humans include EBS with muscular dystrophy (EBS-MD) and EBS with pyloric atresia (EBS-PA). KRT5, KRT14, BPAG1 and PLEC are the genes most commonly affected in cases of basal EBS in humans; variants in PLEC only account for about 8% of the cases. A number of variants in human PLEC have been deemed responsible for EBS and the location of the variant will dictate the severity of disease. EBS-MD is characterized by mild to subtle blistering and nail dystrophy early in life with variable onset muscular weakness (i.e. congenital to as late as the 4th

Figure 3. Electron microscopy of skin from Eurasier dog with epidermolysis bullosa simplex. (a) Eurasier puppy with separation in the basement membrane zone. Note the electron-dense hemidesmosomes (arrowheads) as compared with hemidesmosome in an aged matched control dog (b). Bar = 2 μm. (c) At higher magnification the hemidesmosomes are cone shaped (arrowheads) with loss of distinction between inner and outer plaques. (d) Normal hemidesmosomes from an age-matched control dog showing distinct inner and outer plaques (arrowheads). Bar = 0.5 μm.
decade). EBS-PA has a more severe congenital phenotype (often lethal) with severe skin blistering and pyloric or duodenal atresia. It has been hypothesized that the pyloric stenosis is related to localized blistering and chronic inflammation. Like the Eurasier puppies, the severe EBS-PA phenotype may result from plectin variants that affect the rod and carboxy domain and thereby impair binding to keratin and possibly to integrin β4 as an alternative binding site.4,5

In the affected dogs, the hemidesmosomes were abnormally electron dense and globular to ovoid with thickening of the connecting tonofilaments. The thickening of keratin may represent a compensatory change due to the truncated protein and failure to cross-link. Furthermore, immunofluorescence demonstrated a marked decrease to absence in plectin staining. This finding led to the discovery of the candidate gene. However, based on the C-terminal location of the epitope in the polyclonal antibody, one would predict a complete lack of plectin staining. The light patchy staining is most likely explained by cross-reacting amino acid sequence homology with other basement membrane proteins.

Based on light microscopy and ultrastructural clefting, EB in this group of Eurasier puppies was initially classified as JEB, and the more severe form, Herlitz (now termed JEB generalized severe). Identification of the PLEC variant led to the appropriate categorization as basal EBS: (i) the subcellular location of the defect lies in the basal keratinocyte; (ii) clinical features were generalized; (iii) the disorder was shown to have an autosomal recessive

Figure 4. Indirect immunofluorescence staining for plectin in epidermolysis bullosa (EB). Samples from the oral cavity, pawpad and haired skin from a normal dog and three EB-affected Eurasier puppies were immunostained for plectin on the same day. Whereas the staining for plectin was strong, linear and continuous at the basement membrane zone, that of the three Eurosier puppies was very faint and often patchy or absent. 20x
mode of inheritance; and (iv) the variant was discovered in PLEC and resulted in perturbation of the target protein plectin. This form of EBS is not characterized by cytolsis of basal keratinocytes as seen in EBS due to variants of keratin 5 and 14.2 Light microscopy is unable to distinguish severe JEB from basal plectin-associated EBS.

The Eurasier puppies had severe lesions confined to the skin and oral cavity as documented on complete post-mortem examination. Weakening of the dermo-epidermal integrity lead to blistering and skin sloughing in areas prone to frictional trauma (e.g. oral cavity, genitalia, paw pads). Sloughing of the oral mucosa may have led to nutritional deprivation as evidenced by the small size as compared to the littersmates. It is unknown if the dogs eventually would have developed either PA or MD, as they were humanely euthanized at 20 days of age.

Treatment of the various forms of EB is similar: wound care, preventing and treating infection, as well as supportive care including pain management and nutritional support. A few clinical trials have been performed or are underway in humans.7 This is the first canine model of EBS in which a variant in PLEC has been demonstrated. Studies herein may advance the understanding of PLEC and EBS pathogenesis and provide an avenue to explore new therapies such as topical protein replacement, viral vector gene therapy, small interfering (si)RNAs or gene editing by Clustered Regularly Interspaced Short Palindromic Repeats (referred to as CRISPR).

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References


Supporting Information

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

Table S1. Antibodies used for indirect immunofluorescence for basement membrane proteins.

Table S2. Primers and restriction fragment lengths for detection of disease-associated PLEC non-sense variant.

Table S3. Single nucleotide polymorphisms identified in PLEC exons by sequencing DNA from an affected Eurasi dog.

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Hypotheses/Objectives – Décrire une nouvelle maladie de clivage qui apparaît spontanément dans une portée de chiots.

Sujets – Deux femelles et un mâle chiots de 20 jours Eurasier, issus d’une portée de six, ont été présentés pour évaluation de défaut de croissance puis euthanasie liée au pronostic réservé. Les chiots présentaient des ulcères sur les lèvres, la langue, le planum nasal, les coussinets et l’abdomen.

Résultats – L’immunomarquage sur tissus congelés des protéines de la membrane basale a révélé une coloration faible à absente, en patch pour la plectine comparé avec une forte coloration linéaire pour les chiens normaux. Ultrastructuralement, les hémidesmosomes étaient irréguliers et avaient perdus leur distinction entre les plaques internes et externes. L’analyse du pédigreé a supporté le mode de transmission autosomal récessif. Un codon stop prématuré a été découvert dans l’exon 27 de PLEC qui résultait de la production de la protéine sévèrement tronquée.

Conclusion – L’étude décrit le premier EBS spontané documenté avec un variant de PLEC chez les animaux domestiques.

Resumen
Introducción – la plectina, una proteína de gran tamaño de enlace encontrada en muchos tejidos, actúa para conectar los componentes del citoesqueleto entre ellos. En la epidermis, plectina une filamentos intermedios de queratina a hemidesmosomas. Una deficiencia de plectina en la piel conduce a la formación de ampollas en la capa basal y causa la enfermedad epidermolisis bullosa simplex (EBS).

Hiptótesis / Objetivos – Describir una nueva enfermedad vesicular que surgió espontáneamente en una camada de cachorros.

Animales – Dos hembras y un macho de 20 días de edad de raza Eurasier, de una camada de seis, se presentaron para evaluación por falta de crecimiento y luego fueron sacrificados debido a un mal pronóstico. Los cachorros tenían úlceras en los labios, la lengua, plano nasal, almohadillas de las patas y el abdomen.

Resultados – la inmunodetección en piel congelada de las proteínas de la membrana basal reveló tinción irregular y débil a ausente para plectina en comparación con una fuerte tinción lineal en perros normales. Ultraestructuralmente, los hemidesmosomas eran de forma irregular con una pérdida de la diferenciación entre las placas interior y exterior. El análisis genealógico implica un modo de herencia autosómico recesivo. Un codón de parada prematuro fue descubierto en el exón 27 de PLEC que resultó en la producción de una proteína severamente troncada.

Conclusion – El estudio describe la primera EBS espontánea documentada asociada con un variant de PLEC defectuosa en animales domésticos.

Zusammenfassung


Schlussfolgerung – Diese Studie beschreibt die erste dokumentierte spontane EBS im Zusammenhang mit einer PLEC Variante bei Haustieren.

要約
背景 – プレクチンは大型の接着蛋白として様々な組織に存在し、細胞骨格同士を連結する装置として働く。表皮において、プレクチンはケラチン中間フィラメントとヘモデスモームを結合している。皮膚におけるプレクチンの欠陥は、基底層の水疱形成へとつながり、単純型表皮水疱症（EBS）を引き起こす。

仮説/目的 – 同胞子の子犬に自然発症した、新規の水疱形成性疾患を報告すること。

供与動物 – 6頭の同胞子のうち、20日齢の雄2頭および雌1頭のユーラシアの子犬が成長不全の精査のために来院し、子犬不良のために安楽死された。子犬は、口唇、舌、鼻鏡、掌および腹部に水疱を認めた。

結果 – 凍結皮膚を用いた基底皮膚の免疫標識にて、プレクチンは、正常犬の強い粉状の染色性と比べて、斑状
EBS in Eurasier dogs

Resumo
Contexto – A plectina, grande proteína de ligação encontrada em diversos tecidos, atua na ligação de componentes do citoesqueleto entre si. Na epiderme, a plectina liga a filamentos intermediários de queratina a hemidesmosomos. A deficiência de plectina na pele leva à formação de vesículas na camada basal e à doença epidermolise bolhosa simples (EBS).

Hipótese/objetivos – Descrever uma nova doença vesicular que surgiu espontaneamente em uma ninhada de filhotes de cães.

Animais – Duas fêmeas e um macho da raça Eurasier com 20 dias de vida, oriundos de uma ninhada de seis filhotes, foram apresentados para avaliação de deficiência de desenvolvimento e, posteriormente, eutanasiados devido ao mau prognóstico. Os filhotes apresentavam úlceras nos lábios, língua, plano nasal, coxins e abdômen.

Resultados – Imunomarcação de proteínas da membrana basal de fragmentos de pele congelados, revelaram marcação irregular e fraca a ausente para plectina, quando comparada com a marcação forte e linear em cães normais. Ultraestruturalmente, os hemidesmosomos tiveram formato irregular e perda da distinção entre placas internas e externas. Análise do pedigree corroborou com a existência de herança autosômica recessiva. Um códon de parada prematura foi descoberto no exon 17 de PLEC que resultou na produção de uma proteína intensamente truncada.

Conclusão – O estudo descreveu o primeiro relato de EBS associada à variante PLEC em animais domésticos.