Familial footpad hyperkeratosis and inheritance of keratin 2, keratin 9, and desmoglein 1 in two pedigrees of Irish Terriers

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Objective—To investigate the possibility that variants in the acidic or basic keratin genes or in desmoglein 1 may cause the clinical manifestation of familial footpad hyperkeratosis in Irish Terriers.

Animals—11 dogs belonging to 2 related affected pedigrees of Irish Terriers.

Procedure—Genomic DNA was extracted from blood samples obtained from each dog. The DNA markers linked to the genes keratin 2, keratin 9, and desmoglein 1 were amplified by use of a polymerase chain reaction technique, and length of the products was determined by use of an automatic DNA analyzer.

Results—All tested markers yielded information. None of the markers (genotype) cosegregated with the clinical status of the dogs (phenotype) in the 2 pedigrees.

Conclusions and Clinical Relevance—Mutations in the genes encoding keratin 2 and 9 as well as desmoglein 1 are highly unlikely to be the primary cause of familial footpad hyperkeratosis in Irish Terriers. (*Am J Vet Res* 2003;64:715–720)

Familial footpad hyperkeratosis (also known as corny feet) has been reported in Irish Terriers[1] and the Dogue de Bordeaux.[2] Individually affected dogs have been observed among several related Kerry Blue Terriers, Labrador Retrievers, Golden Retrievers, and mixed-breed dogs.[3] Affected dogs develop severe hyperkeratosis of all footpads, resulting in hard, cracked, abundant keratin tissue by the time the dogs are 5 to 6 months old. Secondary fissures and bacterial infections may subsequently cause severe lameness. Concurrent abnormal claw development has been observed in Irish Terriers.[1]

Histopathologic characteristics of biopsy specimens obtained from the footpads include papillate epidermal hyperplasia with diffuse orthokeratotic hyperkeratosis and fusion of the conical papillae by keratin.[2] However, typical amounts and size of keratin filaments, keratohyaline granules, and lamellar bodies are seen in electron micrographs.[2]

Differential diagnoses include secondary digital (ie, nasodigital) hyperkeratoses associated with distemper,[3] leishmaniosis,[4] zinc-responsive dermatosis,[5] or autoimmune diseases.[6,7] Idiopathic forms of nasodigital hyperkeratosis can be seen in older dogs.[8]

Although it has been suspected that there is a hereditary basis of familial footpad hyperkeratosis in Irish Terriers, the exact mode of inheritance has not been established. Evidence for an autosomal recessive mode of inheritance was reported in 1 retrospective study.[1] By reviewing the pedigrees of 16 afflicted dogs, the authors of that study did not find a sex predilection, but they could establish a good correlation between the statistical estimation of the frequency of affected dogs in affected families and the expected frequency for an autosomal-recessive condition. In humans, several autosomal-dominant, hyperkeratotic skin diseases are associated with mutations in keratin genes.[9,10] For example, epidermolytic hyperkeratosis is linked to mutations in genes encoding keratin 1 or 10,[11-14] whereas ichthyosis bullosa of Siemens is caused by mutations in the keratin 2e gene.[15] Affected patients have hyperkeratotic plaques on the extremities. Mutations in the gene encoding keratin 9 are likely the underlying cause of so-called epidermolytic palmoplantar keratoderma, which is exclusively restricted to the epidermis of the soles and palms.[16,17] Finally, defects in the gene encoding keratin 16 are responsible for focal nonepidermolytic palmoplantar keratoderma,[18] whereas a mutation in the gene coding for **desmoglein 1 (DSG 1)** has been identified as the cause of striate palmoplantar keratoderma.[19] The latter is characterized by hyperkeratotic thickening of the skin on the soles and palms.[19] Because of the clinical similarity with some hyperkeratotic skin disorders in humans, keratin 2, keratin 9, and DSG 1 can be regarded as candidate genes for familial footpad hyperkeratosis of Irish Terriers. By using knowledge about the location of these genes in the canine genome[20-22] and polymorphic markers within or in close proximity to these genes, we investigated the possibility that variants in the acidic or basic keratin genes or DSG 1 may be the cause of this clinical manifestation in Irish Terriers.

Materials and Methods

Animals—Dogs that belonged to 2 pedigrees of Irish Terriers registered in the Dutch Irish Terrier Club were used in the study. The 2 pedigrees had a common sire and consisted of 11 dogs (6 males and 5 females) born between November 1992 and August 1998. Physical examination performed in September 1999 did not reveal abnormalities other than footpad hyperkeratosis. This hyperkeratosis had been evident for 5 to 7 months (mean, 6 months). All footpads were affected in 3 dogs. These dogs had a preference for walking on soft surfaces. Another dog had only slight hyperkeratosis on several, but not all, footpads; therefore, this dog was classified as clinically doubtful. The remaining 7 dogs did not have alterations of the footpads.

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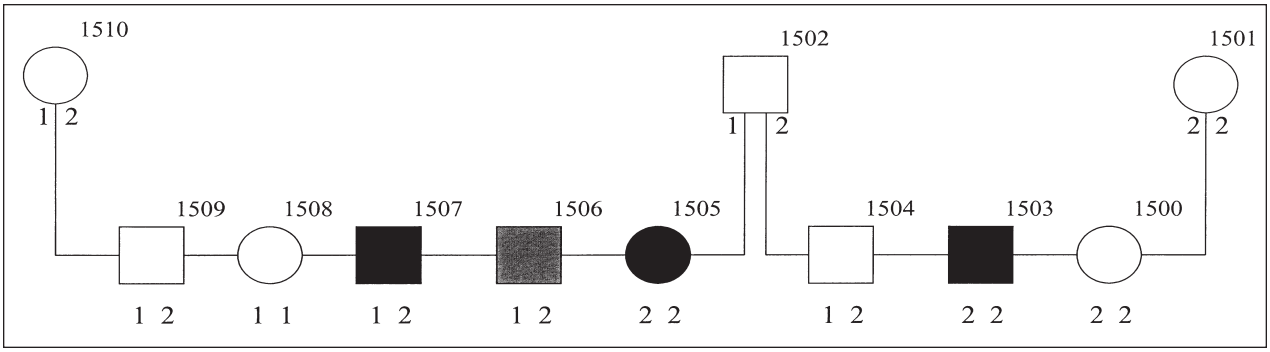

Figure 1—Schematic diagram of the 2 pedigrees of Irish Terriers in which digital hyperkeratosis is segregating for 2 allelles, as determined by results of DNA amplification accomplished by the use of the marker REN01E05. Sex of each dog (male, square; female, circle) is indicated for the F_0 (ie, dogs 1510, 1502, and 1501) and F_1 generations. Two males (black squares) and 1 female (black circle) were affected, and 1 male (gray square) was classified as clinically doubtful. The number above each symbol is the animal's identification, whereas numbers below the symbols indicate the various alleles.

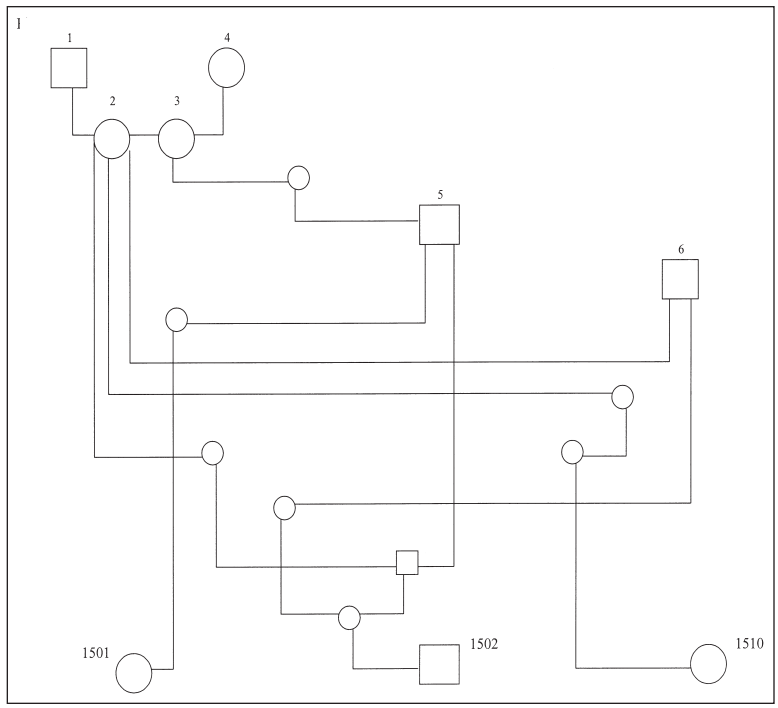

Figure 2—Multiple-generation pedigree depicting the common ancestors (dogs numbered 1 to 6) of the 3 parent dogs of the F_0 generation of Irish Terriers. *See* Figure 1 for key.

The families and clinical status of each dog were characterized **(Fig 1)**. The 3 dogs of the F_0 generation were all related to each other within several generations **(Fig 2)**.

Analysis of genomic DNA—A blood sample (10 mL) was collected via venipuncture from each dog at the time of physical examination. Samples were placed in tubes containing EDTA. Genomic DNA was isolated by the salt-extraction method described by Miller et al[23] and stored at –20°C until use.

Polymerase chain reaction (PCR) technique—A PCR technique was used to amplify DNA. Amplification was performed by use of platinum *Taq* polymerase[a] in a total volume of 15 µL of buffer[b] containing 50 to 100 ng of genomic DNA, 8 pmol of each primer, 2mM deoxynucleoside triphosphate, and 22.5 pmol of $MgCl_2$. The PCR program that was used involved an initial denaturation step of 10 minutes at 94°C followed by 35 cycles of 30 seconds at 94°C, 30 seconds at the annealing temperature, and 30 seconds at 72°C. This was followed by a final extension of 10 minutes at 72°C and a cooling phase of 2 minutes at 20°C. An aliquot of the PCR product was separated on a 2% agarose gel containing ethidium bromide by use of electrophoresis for 1 hour at 120 V in 0.5X Tris-boric acid-EDTA buffer; gels were evaluated by use of UV transillumination. Subsequently, the PCR products were diluted in formamide containing a sizing standard[c] and analyzed on a genetic analyzer with accompanying software.[d]

Primers for PCR analysis—The forward or reverse primer of each pair was labeled with **alkynyl 6-carboxyfluorescein (FAM)** to confer green-yellow fluorescence to the primer, **tetracycline (TET)** to confer orange fluorescence, or **6-carboxyfluorescein hexachloride (HEX)** to confer pink fluorescence. Several primer pairs were purchased for analysis of keratin 9 (forward, REN01E05; reverse, FAM/REN01E05 [annealing temperature, 64°C]),[24,e] DSG 1 (forward, FH2201; reverse, HEX/FH2201 [annealing temperature, 58°C] and forward, REN97N23; reverse, TET/REN97N23 [annealing temperature,

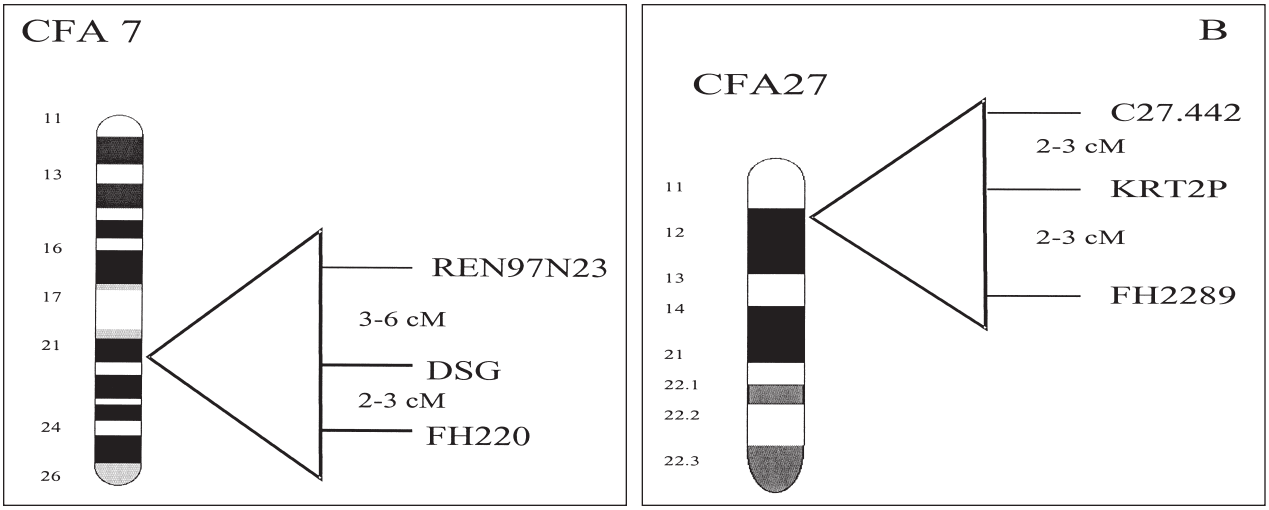

Figure 3—Position of linked polymorphic DNA markers (REN97N23 and FH2201) relative to the desmoglein 1 (DSG 1) gene on canine chromosome 7 (CFA 7; panel A) and markers (C27.442 and FH2289) relative to the basic keratin gene (KRT 2P) on canine chromosome 27 (CFA 27; panel B). Idiograms[22] and the relative location of the markers[20] have been reported elsewhere. Distances between markers and each gene have been converted to number of centiMorgans (cM) by applying the following approximation for dogs[25]: 10 centiRays is approximately 1 to 2 cM. The numbers on the left of the chromosome diagrams indicate the chromosomal regions, bands, and subbands in accordance with the nomenclature described by the International System for Cytogenetic Nomenclature.

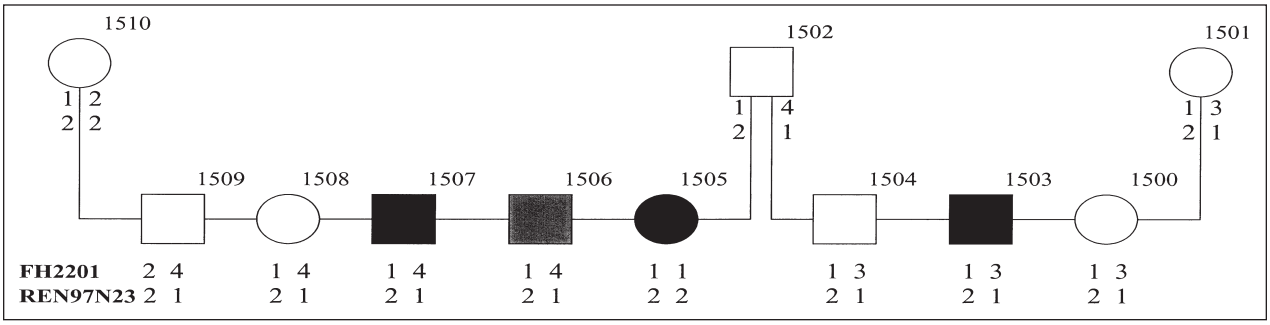

Figure 4—Schematic diagram depicting inheritance of alleles in Irish Terriers as determined by results of DNA amplification accomplished by the use of the markers FH2201 and REN97N23. Two males (black squares) and 1 female (black circle) were affected, and 1 male (gray square) was classified as clinically doubtful. *See* Figure 1 for remainder of key.

58°C]),[22,f,g] and keratin 2 (forward, C27.442; reverse, TET/C27.442 [annealing temperature, 55°C] and forward, FH2289; reverse, FAM/FH2289 [annealing temperature, 53°C]).[20,h,i]

Microsatellite markers—The marker REN01E05 lies within the gene keratin 9.[24] The markers REN97N23 and FH2201 flank DSG 1 on **canine chromosome 7 (CFA 7)**,[22] whereas the markers C27.442 and FH2289 flank keratin 2P on **canine chromosome 27 (CFA 27)**.[20] The chromosomal localization and genetic distances to the genes DSG 1 and keratin 2P have been characterized (**Fig 3**). After typing these DNA markers in each of the dogs in the 2 pedigrees, a statistical genetic analysis was performed to calculate the likelihood of exclusion of the candidate genes. To this end, multiple-point linkage analysis was implemented by use of a computer program.[26,j]

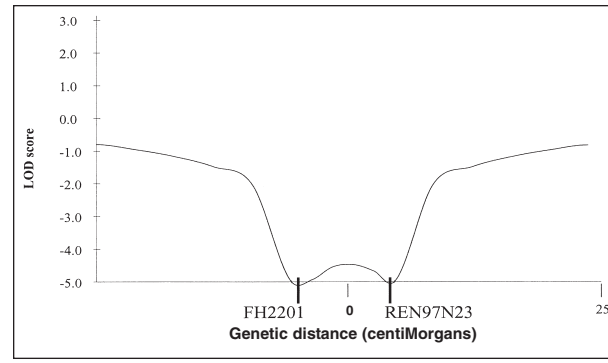

Figure 5—Results for typing of the polymorphic DNA markers FH2201 and REN97N23 in the 2 pedigrees of Irish Terriers. Multipoint linkage analysis for DSG 1 was implemented by use of a gene-mapping program.[26,a] Notice that the logarithm of odds (LOD) score between the 2 markers remains lower than –4.0 throughout.

Results

Analysis of keratin 9—Amplification of DNA by use of primers for intragenetic marker REN01E05 yielded 2 alleles (403 and 409 bp, respectively). Analysis of the inheritance of these 2 alleles in the 2 pedigrees did not reveal a correlation between their distribution (ie, genotype) and clinical status of the dogs (ie, phenotype; Fig 1). Affected dogs in the F_1 generation carried the genotypes (2, 2) and (1, 2). In the F_0 generation, 3 other dogs had the same genotypes but were not affected. In view of the large number of recombinants between this intragenetic marker and the disease locus, further statistical analysis was not performed.

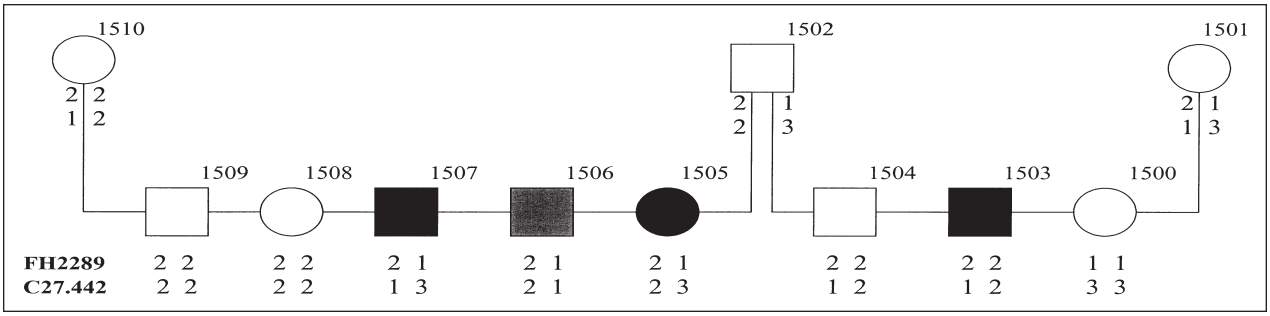

Figure 6—Schematic diagram depicting inheritance of the alleles in Irish Setters as determined by results of DNA amplification accomplished by use of the markers FH2289 and C27.442. Two males (black squares) and 1 female (black circle) were affected, and 1 male (gray square) was classified as clinically doubtful. *See* Figure 1 for remainder of key.

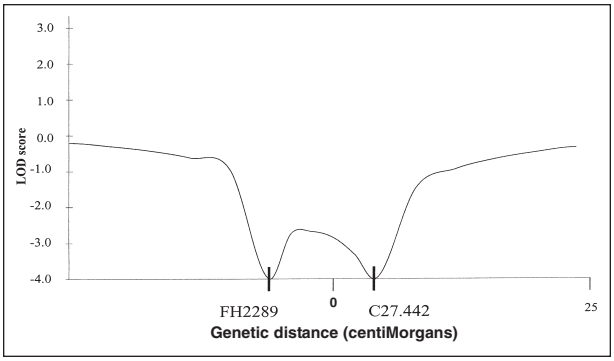

Figure 7—Results for typing of the polymorphic DNA markers FH2289 and C27.442 in the 2 pedigrees of Irish Terriers. Multipoint linkage analysis for DSG 1 was implemented by use of a gene-mapping program.[25,a] Notice that the LOD score between the 2 markers remains lower than –2.3 throughout.

Analysis of DSG 1—The genetic marker FH2201 is located immediately proximal to the gene encoding for DSG 1, and the genetic marker REN97N23 is located immediately distal to this gene on CFA 7 (Fig 3).[22] The DNA amplification for FH2201 yielded 4 alleles of 155, 159, 167, and 171 bp, respectively. Amplification with REN97N23 yielded 2 alleles of 229 and 235 bp, respectively. A relationship between genotypes and phenotypes could not be established with regard to these markers (**Fig 4**). For FH2201, an affected dog in the F_1 generation carried the genotype (1, 3). In the F_0 generation, another dog had the same genotype but was not affected. One dog was homozygous (1, 1), and it could be concluded that this genotype correlated with the disease. However, under this presumption, it cannot be explained why another dog, which had the genotype (1, 4), would also be affected, but a female sibling that had the same genotype would not. For REN79N23, affected dogs in the F_1 generation had the genotypes (2, 1) and (2, 2). The same genotypes were found in nonaffected dogs in the F_0 and the F_1 generations. As expected, we did not detect recombination between the 2 DNA markers. The maximal **logarithm of odds (LOD)** score between the polymorphic markers FH2201 and REN97N23 was $10^{-4.3}$ (**Fig 5**).

Analysis of keratin 2—The 2 flanking markers (ie, C27.442 and FH2289) were tested in the DNA of our test population and yielded information (**Fig 6**). For C27.442, 3 alleles of 292, 296, and 300 bp, respectively, were observed. For FH 2289, 2 alleles of 159 and 165 bp, respectively, were observed. Also, these genotypes did not correlate with the clinical phenotype of the population of dogs examined. Similar to the situation for C27.442, affected dogs in the F_1 generation carried the genotypes (1, 2), (2, 3), and (1, 3), respectively. In the F_0 generation, 3 other dogs had the same genotypes but were not affected. For FH2289, affected dogs in the F_1 generation carried the genotypes (2, 2) and (2, 1). Also, dogs in the F_0 generation had the same genotypes but were not affected. As expected, we did not detect recombination between the 2 DNA markers. The maximal LOD score between the polymorphic markers FH2289 and C27.442 was $10^{-2.6}$ (**Fig 7**).

Discussion

A diagnosis of hereditary digital hyperkeratosis is usually based on signalment, results of physical examination, and clinical history of dogs more than 4 to 6 months of age.[1,3] Initial signs are associated with the development of smooth, parchment-like footpads. During later stages, severe hyperkeratosis of the footpads of all feet are observed. Affected Irish Terriers also have an increase in growth and change in transverse profile of the nails. Such dogs prefer to walk on soft surfaces.[1] In the 2 pedigrees reported here, 3 dogs met these clinical criteria. One dog had only moderate hyperkeratosis, and it was not lame or unwilling to walk. Therefore, the dog was classified as clinically doubtful, and the dog was not included when considering the interpretation of segregation of the tested markers.

Binder et al[1] provided evidence that digital hyperkeratosis is inherited in an autosomal-recessive mode. The purpose of the study reported here was to investigate whether the disease could be linked to certain genes. To this end, knowledge of similar diseases and the responsible genetic mutations in humans was extrapolated to dogs. In the past, this approach has led to the identification or exclusion of genes responsible for other hereditary diseases in different species, such as Ehlers-Danlos syndrome in humans, dermatosparaxis in cattle,[27] or digital hyperkeratosis in the Dogue de Bordeaux.[28]

The genes keratin 2, keratin 9, and DSG 1 were selected on the basis that hereditary hyperkeratotic skin diseases in humans that clinically resemble hereditary digital hyperkeratosis in Irish Terriers have been

associated with mutations in these genes. Products of these genes are subunits of the cytoskeleton (keratin 2 and 9) and desmosomes (DSG 1). These structures are essential for cohesion within the epidermis; desmosomes represent cell junctions in epithelial cells.[29] The gene DSG 1 encodes for DSG 1, which, in combination with desmoglein 3 and desmocollins 1 and 3, represents the adhesive proteins of the desmosomes. They share a similar structure in that the extracellular part is connected with the intracellular, plaque-protein binding sites through a single transmembrane region. The binding sites for plaque-proteins provide the connection to intermediate filaments of the cytoskeleton (cytokeratins and actin). By analysis of the pedigrees of the dogs reported here, we were able to exclude the DSG 1 gene as a cause for the disease with a high probability (> 1 to 10^4, which corresponds approximately to a value of $P < 0.001$).

For keratin 9, the tested marker REN01E05 is located within the gene,[24] and analysis of the results reported here indicates that it is highly unlikely that the keratin 9 gene causes the disease. Assuming that the sequence of the keratin genes within the acidic keratin cluster in dogs is similar to the corresponding sequence in humans, the following distribution would result from use of a gene-mapping program[30]: keratin 9 in humans identified with the marker AFMa119yd1 would be localized at coordinate 2158 **centiRay** (**cR** $_{10,000}$). A centiRay is a statistical number used in the physical mapping of genes; 1 cR corresponds to approximately 1 to 2 million bp. Keratin 13 is located immediately proximal to coordinate 2151; keratin 9 and keratin 15 have the same coordinates (ie, 2158), because the distance between them is so small that the sequence of these 2 genes could not be determined. Keratin 13 and keratin 15 are at a genetic distance of < 0.1 **centiMorgans (cM)** from keratin 9 (equal to a probability of recombination of 0.1%). Thus, keratin 13 and keratin 15 are also highly unlikely to be linked to digital hyperkeratosis.

Miller et al[20] have localized the keratin 2p gene to canine chromosome 27 (ie, CFA 27) by use of radiation hybrid mapping. The gene has been placed between the markers FH2289 and C27.442. Analysis of the pedigrees of the dogs reported here enabled us to exclude the keratin 2p gene as a cause for the disease with a high probability (> 1 to 300, which corresponds approximately to a value of $P = 0.003$). The assigned region of CFA 27 corresponds to human chromosome 12q, analogous to the region where the human keratin 2e gene has been mapped.[31] Assuming the arrangement of genes within the basic keratin gene cluster is similar in dogs and humans, this would imply that keratin 2e is located next to keratin 2p in both species and that the basic keratin genes (ie, 1, 4, 5, 6A, 8, and 18) are highly likely to cosegregate with keratin 2p, because the genetic distance between these genes would be < 1 cM in dogs, similar to the distance reported in humans.[32] Therefore, it is unlikely that these genes would be candidates to cause digital hyperkeratosis.

It could be argued that the underlying assumption of 1 mutational event causing this type of hyperkeratosis in the footpads of Irish Terriers is an oversimplification. Recent advances in clinical epidemiologic techniques may shed light on this issue. The molecular basis of von Willebrand's disease has been elucidated for a number of breeds of dog.[33,34] In every breed studied to date, a single mutation in the gene for von Willebrand's factor has been found in all affected dogs, and in most cases, the mutation is unique for that breed. We analyzed the linkage disequilibrium between the mutation of the von Willebrand gene and linked DNA markers in 2 breeds and found a complete linkage disequilibrium. The same complete linkage disequilibrium was found for the gene causing copper toxicosis in Bedlington Terriers and a linked DNA marker.[35] Thus, in situations where this could be assessed, the hypothesis for a multiple-mutational event was rejected.

Many factors that control the process of keratinocyte proliferation and differentiation must be in finely tuned equilibrium to maintain epidermal homeostasis.[3] In response to injury or inflammation, the balance is shifted toward proliferation, and it returns to normal with wound healing. Keratin proteins emerging from prekeratin in the stratum basale and stratum spinosum represent the major barrier between animals and the environment. In addition to these keratin proteins, other factors are involved.[36-39] Extracellular signals can influence epidermal turnover through various second-messenger systems. Intrinsic factors, such as epidermal growth factor, fibroblast growth factors, arachidonic acid metabolites, proteolytic enzymes, and various hormones, also play a role in this process. Another important factor is the activity of the enzyme ornithine decarboxylase, which is essential for the synthesis of certain polyamines (ie, putrescine, spermidine, or spermin) that stimulate epidermal proliferation. Because digital hyperkeratosis is characterized by a marked orthokeratotic hyperkeratosis with loss of cohesion in the stratum corneum without ultrastructural aberrations in the lower layers of the epidermis, it could be speculated that the process of cornification is altered. In fact, any element of epidermal proliferation and differentiation could be the underlying cause of the digital hyperkeratosis.

The disturbance also may affect formation of the cell envelope (cross-linking of certain cytosolic proteins, such as loricrin, involucrin, keratolinin, pancornulin, and corifin, by transglutaminases). In humans, a group of hereditary palmoplantar keratodermas associated with a mutation in the loricrin gene has been described.[40]

The study reported here revealed that the genes encoding for keratin 2, keratin 9, and DSG 1 are highly unlikely to cause hereditary digital hyperkeratosis in Irish Terriers, because they do not cosegregate with the disease. Keratin 1, 4, 5, 6A, 8, 13, 15, and 18, which have been mapped in close proximity to these genes, can also be regarded as an unlikely cause, as determined by linkage analysis.[30,32] Additional studies, preferably a full genome scan for the canine genome, are indicated.

[a]Platinum *Taq* polymerase, Gibco, Breda, The Netherlands.
[b]BRL, Gibco, Breda, The Netherlands.

[c]GS-500 sizing standard, Applied Biosystems, Foster City, Calif.
[d]ABI 310 genetic analyzer with GENESCAN software, Applied Biosystems, Foster City, Calif.
[e]REN01E05F-FAM/REN01E05R, Invitrogen, Paisley, Scotland.
[f]FH2201F-HEX/FH2201R, Invitrogen, Paisley, Scotland.
[g]REN97N23F-TET/REN97N23R, Invitrogen, Paisley, Scotland.
[h]C27.442F-TET/C27.442R, Invitrogen, Paisley, Scotland.
[i]FH2289F-FAM/FH2289R, Invitrogen, Paisley, Scotland.
[j]Genehunter 2.1. Available at: www.fhcrc.org/Labs/kruglyak/Downloads/index.html. Accessed.

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