Institutionen för husdjursgenetik

Evaluation of Kallikrein 7 (KLK7) and Serine Protease Inhibitor Kazal-type 5 precursor (SPINK5) as candidate genes in Canine Atopic Dermatitis in Boxer and West Highland White Terrier

by

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Övrigt: Canine, Atopic Dermatitis, SPINK5, Kallikrein 7

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Abbreviations

AD	Atopic Dermatitis
APC	Antigen-presenting cells
bp	Basepair/s
CAD	Canine Atopic Dermatitis
CAFR	Cutaneous Adverse Food Reactions
dNTP	deoxyribonucleoside triphosphate
FcεR1	High-affinity receptors on the surface of mast cells (and basophils), which binds the Fc region of IgE.
indel	Insertion/deletion polymorphism
IgE	Immunoglobulin E
kb	Kilobase/s
KLK5	Kallikrein 5
KLK7	Kallikrein 7
LEKTI	Lympho-epithelial Kazal-type related inhibitor
LD	Linkage Disequilibrium
MHC	Major Histocompatibility Complex
NTS	Netherton Syndrome
PCR	Polymerase Chain Reaction
SCCE	Stratum Corneum Chymotryptic Enzyme
SCTE	Stratum Corneum Tryptic Enzyme
SNP	Single Nucleotide Polymorphism
SPINK5	Serine Protease Inhibitor Kazal-type 5 precursor
TcR	T-cell Receptor
WHWT	West Highland White Terrier

Abstract

Atopic Dermatitis is a genetically predisposed allergic skin disease that affects both humans and dogs. The disease has a complex background influenced by both genetic and environmental factors. The genetic origin is still largely unknown but genetic screens have indicated several different genes or different groups of interacting genes in the aetiology of the disease. These genes are involved, together with environmental factors, in processes that lead to the expression of the clinical phenotype. In humans the genetic background is most likely more complex than in many dog breeds due to the intense inbreeding that many dog populations have experienced. Certain dog breeds are more predisposed to develop Canine Atopic Dermatitis (CAD) than others and high-risk breeds are for instance Boxer and West Highland White Terriers (WHWT). Case and control animals from these two breeds were included in this study where two candidate genes for CAD were evaluated. The genes Kallikrein 7 (KLK7) and Serine Protease Inhibitor Kazal-type 5 precursor (SPINK5) have, based on studies that have implicated these genes in atopic diseases, been selected as candidate genes for CAD.

Two different kinds of genetic markers were used in the evaluation; three microsatellites in the near vicinity of the gene were chosen for the evaluation of KLK7, and single-nucleotide polymorphisms (SNPs) were searched for in one intron of SPINK5. No informative SNPs in the SPINK5 gene were found in WHWT (except for individual mutations) but four SNP loci were found within the sequenced area of 1,500 base pairs in Boxer. These SNP alleles were connected into two haplotypes but neither of the haplotypes showed any association with the disease phenotype. However, additional test individuals as well as more polymorphic markers, which are better spread out within and around the gene, are needed for an improved evaluation of SPINK5´s involvement in the disease in these two dog breeds. In KLK7, the three microsatellite-markers were both quite polymorphic (except for one locus in WHWT) and well distributed within and around the gene, and the data conclusively show lack of association between the gene and the disease phenotype.

Introduction

The objective of this study was to evaluate whether two chosen genes were associated with Canine Atopic Dermatitis (CAD) in the two high-risk breeds West Highland White Terrier (WHWT) and Boxer. The disease CAD will be discussed in the first part of this report followed by a short summary of the most important components in the antigen-specific immune response. Earlier studies on human and mice have shown that the two genes, Kallikrein 7 (KLK7) and Serine Protease Inhibitor Kazal-type 5 precursor (SPINK5) could be involved in atopic dermatitis. In addition, the genes have shown to have important functions in the epidermis. These genes have therefore been chosen as candidate genes for CAD in this study and are described in detail later in this report. The design of the project was to search for informative genetic markers in the near vicinity of the two candidate genes and seek possible associations between these markers and the disease phenotype. Case and control animals were compared to each other within each of the two dog populations.

The complexity of Atopic Dermatitis

Atopic dermatitis (AD) has a complex background with both genetic and environmental influences. Complex diseases such as this are described as multifactorial since they are dependent on an interaction between multiple major and minor genes and also important environmental factors, in particular the exposure to different allergens (Barnes & Marsh, 1998). Numerous environmental allergens have shown to be involved in the pathogenesis of AD. These include dust and storage mite antigens, house dust, grass, tree and weed pollens, mould spores, epidermal antigens, insect antigens and various antigens such as kapok (Hill & DeBoer, 2001).

The nature of AD can also be described as heterogenic, which means that different genes, or different groups of interacting genes, can lead to the expression of the clinical phenotype. A great variety of mutations are likely to exist among the major genes involved in the disease. These modulate the gene functions differently and together with a different mixture of environmental factors they will result in a diversity of disease expressions (Barnes & Marsh, 1998). This is typical for a complex disease and explains the large variety of symptoms described in human, as well as in canine patients, with atopic diseases.

Dogs have a history of intense inbreeding, which has resulted in low genetic diversification within different breeds of dogs. Furthermore, specific genetic disorders are predisposed in certain dog populations and CAD is one such clear example. Due to the small genetic diversity, CAD should in one particular breed have a more homogeneous origin compared with AD in many human populations that are more genetically diverse. The chance to find a certain gene or genes involved in the disease increases if the population has a low genetic diversity because the genetic cause should then be identical by descent within that certain breed. Another help in searching for genes involved is a high disease incidence within the selected dog population. High-risk breeds are for instance WHWT, Bullterrier and Boxer (Nødtvedt *et al*, in press).

Canine Atopic Dermatitis, definition and historical background

CAD is defined as a genetically predisposed inflammatory and pruritic allergic skin disease with characteristic clinical features, most commonly associated with IgE antibodies to environmental allergens (Olivry *et al*, 2001).

The first published description of canine atopic disease was in 1941 on a dog with seasonal signs of typical allergic symptoms such as sneezing, tearing with conjunctival infection (conjunctiva is the mucus membrane on the inner surface of the eye lids) and nasal blocking. A similar well-documented case was reported in 1960 with conjunctivitis (inflammation of conjunctiva), lacrimation (secretion of tears) and pruritus (itchiness). Later in the 1960s and early 1970s, reports of multiple cases of canine atopic diseases began to appear in the literature and inflammatory skin lesions were established as a manifestation of canine atopy. These studies reported the presence of skin diseases but no specific clinical criteria for diagnosis of atopic dermatitis or other allergic symptoms in dogs were described. Despite the lack of rigid criteria for diagnosis it was during these two decades that canine atopic diseases were recognized to affect primarily the skin (Griffin & DeBoer, 2001).

The first published reports aimed at quantifying the frequency of clinical signs in dogs appeared in the 1980s. However, the criteria for diagnosis of CAD were still lacking and were in some cases not even specified. For the most part, the only requirement was the presence of pruritus (Griffin & DeBoer, 2001).

In 1986, Willemse proposed a list of clinical criteria for a definitive diagnosis of CAD. In 1998, Prélaud *et al*, evaluated these criteria and made an update. Though both these lists are helpful in determining if a dog shows signs that are consistent with CAD, they are not completely reliable. Other diseases such as food reactions and scabies (a contagious itch or mange, i.e. eczematous inflammation and loss of hair, which is caused by parasitic mites such as *Sarcoptes scabiei* (Webster´s homepage) can satisfy the criteria for CAD (Griffin & DeBoer, 2001).

Symptoms and diagnosis of Atopic Dermatitis

AD is a highly pruritic chronic inflammatory skin disease. In humans, the first clinical signs usually present during early infancy or childhood but can persist or start in adulthood (Leung & Bieber, 2003).

In dogs, clinical signs are usually first detected between 6 months and 3 years of age. The initial signs of CAD can be seasonal or non-seasonal depending on the allergens involved. 42-75% of the dogs with the disease have seasonal initial signs and approximately 80% of these are symptomatic in the spring to fall leaving the rest symptomatic in the winter (Griffin & DeBoer, 2001). However, the majority of dogs with CAD will eventually exhibit non-seasonal signs as the disease progress. A typical dog with atopic dermatitis shows signs of pruritus of the face, ears, paws, extremities, and/or ventrum. It has not yet been established whether uncomplicated CAD alone can result in primary lesions. However, secondary lesions caused by chronic pruritus and trauma, chronic inflammation and concurrent secondary infections or microbial overgrowth are commonly reported in CAD (Griffin & DeBoer, 2001).

The inclusion criteria for CAD described by Willemse in 1986 are shown in Table 1. The patient must have at least three of the major diagnostic features as well as three of the minor features fulfilled after the exclusion of other causes of pruritus in order to obtain the diagnosis. Dermatologists have, in their clinical evaluation of potentially allergic dogs, used this list of criteria extensively. Unfortunately, these criteria have never been evaluated regarding sensitivity, specificity and accuracy for diagnosis of CAD (DeBoer & Hillier, 2001).

Table 1. Willemse`s criteria for the diagnosis of Canine Atopic Dermatitis (Willemse *et al*, 1986).

Major diagnostic features of canine atopic dermatitis

- pruritis
- a typical morphology and distribution
  - (i) facial and/or digital involvement or
  - (ii) lichenification of the flexor surface of the tarsal joint and/or the extensor surface of the carpal joint
- chronic or chronically-relapsing dermatitis
- an individual or family history of atopy
- presence of a breed predisposition

Minor diagnostic features of canine atopic dermatitis

- onset of symptoms before the age of 3 years
- facial erythema and cheilitis
- bilateral conjunctivitis
- a superficial staphylococcal pyoderma
- hyperhydrosis
- immediate skin test reactivity to inhalants
- elevated allergen-specific IgG
- elevated allergen-specific IgE

By using a survey of seven veterinarians examining 96 patients a new list of five major criteria for the disease was proposed by Prélaud *et al*, in 1998, see Table 2 (DeBoer & Hillier, 2001). The presence of three features from the list resulted in diagnostic accuracy of approximately 80% according to the study (DeBoer & Hillier, 2001).

Table 2. Diagnostic criteria for Canine Atopic Dermatitis defined by Prélaud in 1998 (DeBoer & Hillier, 2001).

Major diagnostic criteria for canine atopic dermatitis

- onset of clinical signs between 6 months and 3 years
- corticosteroid sensitive pruritus
- ear pinnae erythema
- anterior bilateral erythematous pododermatitis
- cheilitis

Regardless of which exact list is used as a “checklist” for diagnosis there is a general agreement that other differential diagnosis must be ruled out before a diagnosis of CAD can be obtained. The following diagnoses are generally considered as most important to eliminate from the list of the potential primary or secondary problems: flea allergy dermatitis, cutaneous adverse food reactions (CAFR, either hypersensitivity or non-immunological reactions),

scabies or other pruritic mite infestation, pruritic bacterial folliculitis, *Malassezia* dermatitis and less commonly, cornification disorders and contact dermatitis. Dogs with pruritic non-atopic diseases, in particular food hypersensitivity and scabies, could satisfy many of the inclusion criteria (DeBoer & Hillier, 2001). In order to evaluate the potential contribution of CAFR a well-performed hypersensitivity diet trial (at least 8 weeks followed by a provocation period) has to be conducted. This diet trial is not always performed properly or at all, thus hypersensitivity to food may be a part or the whole reason for the symptoms.

CAFR and CAD were not always regarded as two different diseases in the early reports. Some of these publications included food allergens as causes of CAD, and combining food-allergic dogs with those with CAD may have led to inaccurate descriptions of clinical signs of CAD (Griffin & DeBoer, 2001). However, many typical features are common in both CAFR and CAD and there have been reports on a high prevalence of CAD in dogs with CAFR (13-30%) (Hillier & Griffin, 2001b). There is still not enough evidence to support or refute any association between CAD and CAFR in dogs. In humans, on the other hand, there have been many experimental and clinical studies recognizing a clear relationship between food allergens and the development of AD. Between 30 and 40% of infants and young children with AD suffer from concurrent food allergies (Hillier & Griffin, 2001b).

Incidence

AD in humans is a worldwide public health problem with 10-20% of the children affected and 1-3% of the adults (Leung & Bieber, 2003). In the last thirty years the incidence has increased two to three-fold in industrialized countries whereas the rate remains much lower in agricultural countries such as China, Eastern Europe and rural Africa. In addition, there are higher rates in urban regions compared with rural regions of developed countries and the disease is also more common in higher social groups (Leung & Bieber, 2003). The increasing incidence has been closely linked to a higher indoor allergen load, enlarged exposure to noxious pollutants, smaller family sizes, decreased exposure to infection at a young age, decreased microbial load and an increase of urbanized habitats (Hillier & Griffin, 2001a).

The true incidence of CAD is currently unknown. A great difficulty in determining the incidence of CAD is that the owners have different interpretation of what is abnormal and normal scratching, and that will in turn decide whether the dog is even presented to a veterinarian. This means that milder forms of the disease may be underreported and therefore the incidence rate could be underestimated (Griffin & DeBoer, 2001). Another difficulty in evaluating the frequency of the disease is the great problems in giving a correct diagnosis to dogs with allergic symptoms since no definite diagnostic test is available.

With the aim to estimate the incidence rate of CAD as well as evaluating risk factors connected to the disease, Nødtvedt *et al*, studied Swedish dogs insured by the largest animal insurance company in Sweden, Agria. Insurance claims records from Agria were accessed and all dogs with a veterinary care- and life insurance within the years 1995 to 2002 were included in the analysis. The incidence rate of CAD was estimated to 1.7 cases per 1,000 dog years at risk. Breed incidence varied greatly, with the Bullterrier in the top, with 21 cases per 1,000 dog years at risk, followed by the Welsh Terrier, Boxer and the WHWT. Other factors increasing the incidence of CAD (than belonging to a risk breed) were according to the study, urban environment, living in central or southern Sweden or being born in the autumn (Nødtvedt *et al*, in press).

Heritability

In a study by Shaw *et al*, 2004, the heritability of CAD in Golden and Labrador Retrievers was estimated to 0.47 (ranging from 0.13 to 0.81). The results from this study also showed the risk of being affected in relevance to the parents' status. If both parents had CAD, 65% of the offspring displayed CAD signs, 21% to 57% of the offspring developed the disease if one parent was atopic and only 11% of the offspring was classified with CAD if both parents were non-atopic. These results were in agreement with studies made in humans. This shows clearly that CAD has a strong genetic predisposition.

Inflammatory response in cutaneous allergic reactions

Several inflammatory cells have been reported to play a role in the pathogenesis of AD. However, some cell types seem to be of greater importance for the disease than others, these include mast cells, B-lymphocytes, allergen-specific helper T-lymphocytes, Langerhan's cells and dermal dendritic cells (Hill & Olivry, 2001). Below follows a description of these cells in order to briefly picture how the immune system works and how it is involved in the disease development. The antibody IgE, produced by B-lymphocytes, is also an important component in allergic reactions and will be described as well.

Mast cells

Mast cells are found in the connective tissue and mostly in the areas of the body that interface with the environment i.e. skin, lung and gastrointestinal tract. Mast cells synthesize and release inflammatory mediators such as histamine, proteases and cytokines. In an allergic reaction, this process is usually triggered by the interaction between allergens and allergen-specific IgE. These allergen-IgE complexes are then bound to high affinity FcεR1 receptors that are expressed on the mast cell's surface. The mediators are released through a complex series of biochemical reactions involving G-proteins, tyrosine kinases, inositol-triphosphate, protein kinase C, calcium channel activation and rearrangement of the cytoskeleton. Once released into the extra-cellular space, the mast cell mediators assist in interplay between the microvasculature and other inflammatory cells. Although this response has evolved as a protection against parasites, it is often directed towards harmless environmental antigens and thereby contributes to the development of allergic diseases such as AD (Thoday *et al*, 2000).

Antigen-presenting cells

Antigen-presenting cells (APCs), are found in the gastrointestinal tract, the skin, in the lymphoid organs as well as in other tissues where antigen can enter the body. The APCs main functions are to take up antigen, process and present them to T-lymphocytes in a MHC-restricted manner (see below), and provide co-stimulatory signals for activation of naïve T-lymphocytes. The most important of the APCs are the bone marrow-derived cells of the myeloid lineage, e.g. dendritic cells that are found in many tissues. The dendritic cells are the major APC for initiating primary T-cell responses (Coico *et al*, 2003).

Langerhan's cells are immature dendritic cells found in the epidermal layer of the skin. Their main function is to trap and transport protein antigens to the lymph nodes. Langerhans cells mature into lymph node-dendritic cells during their passage to the lymph nodes (Abbas *et al*, 2004).

Lymphocytes

Lymphocytes are one type of white blood cells. They are continuously produced in the bone marrow, circulate in the blood and lymphatic systems and reside in lymphoid organs. There are two main populations of lymphocytes; B-lymphocytes and T-lymphocytes (Goldsby *et al*, 2000).

B-lymphocytes mature in the bone marrow and as they leave the marrow, expression of a unique antigen-binding receptor, which is a membrane-bound antibody molecule, is initiated in each B-lymphocyte. When a B-lymphocyte for the first time encounters the antigen, which matches its membrane-bound antibody, the binding between antigen and antibody causes the B-lymphocyte to proliferate. The cells produced differentiate into memory and effector B-cells, called plasma cells. The fully differentiated plasma cell is committed to secrete large amounts of antibody molecules with specificity for a single antigen that triggered the B-lymphocyte activation (Goldsby *et al*, 2000).

T-lymphocytes mature in the thymus where each cell starts to express particular T-cell surface-marker molecules. The most important effector molecule on T-cells is the antigen-specific T-cell receptor (TcR) that recognizes peptide antigens presented by major histocompatibility complex (MHC) class I or II molecules. The presentation of antigens by MHC molecules to T-cells initiates an antigen-specific immune response. One type of additional marker molecules are co-receptors to the TcR which allows the T-cell to specifically recognize either MHC class I or class II molecules (Goldsby *et al*, 2000). There are two main categories of T-lymphocytes, which can be identified on the basis of their cell surface-markers. The first group, T-helper cells, possesses the CD4 marker, which is a co-receptor for MHC class II molecules. These cells, after activation from an antigen-MHC class II molecule complex, become an effector T-cell that secretes a variety of growth factors called cytokines. The cytokines (Goldsby *et al*, 2000) stimulate B-lymphocytes to produce immunoglobulins, and coordinate the effects of other inflammatory cells (Hill & Olivry, 2001). Variations in produced cytokines result in different types of immune response (Goldsby *et al*, 2000). The other group, the T-cytotoxic cells, has the CD8 marker which is the co-receptor for MHC class I molecules. These cells respond to foreign antigens that are synthesised within cells, i.e. virus particles, and are able to induce cytotoxic effects (Hill & Olivry, 2001).

In a study by Marsella and Olivry, 2001, an increase in CD4 and CD8 T-cells and a predominance of CD4 T-cells in the epidermis was found in lesional skin of dogs with CAD when compared to non-lesional skin of affected dogs and non-affected controls.

IgE antibodies

In an allergic reaction the T-helper cells start secreting cytokines when they are exposed to certain allergens. The cytokines stimulate class switching of the B-cells into IgE-production, which set off the immunological response through the mast cells activation (see above). During an ongoing allergic response, the mast cells of atopic individuals are coated with IgE antibodies specific for the major allergen of an individual. The mast cell coating process of IgE is called sensitization. In non-atopic individuals the mast cells are covered with a variety of IgE in small amounts that do not cause an allergic reaction (Abbas *et al*, 2004).

In a study by Ledin *et al*, 2006, the plasma levels of IgE in dogs were investigated in order to evaluate whether they are affected by age, breed and/or health status. The IgE levels reported in the literature vary greatly. This inconsistency is probably due to the fact that IgE is normally present at very small concentrations in sera thus causing technical difficulties (mainly due to cross-reactivity to IgG). A new detection method, an *in vitro* assay determining specifically the absolute levels of total IgE in dog sera, was introduced in this study.

The IgE levels from 76 adult dogs, healthy or with the diagnosis of CAD, autoimmunity or infections by skin parasites, were measured with the new assay. The levels ranged from 1 to 41µg/ml and showed no correlation to breed or health status. Thus, the levels of total IgE does not seem to predispose a dog to be allergic according to this study (Ledin *et al*, 2006). These results are in contrast with other studies where the presence of antigen-specific IgE was shown to correlate with allergic symptoms (Barret *et al*, 2003, Masuda *et al*, 2002).

The normal IgE-levels in humans are approximately 0.15µg/ml and in allergic humans up to 1.5µg/ml. The higher IgE-levels in dogs may be explained from the dog's higher exposure of allergens and parasites in their environment (Ledin *et al*, 2006). In Ledin's study the levels of 33 juvenile dogs showed that the IgE levels seem to increase with age, indicating that the exposure of antigens affect the IgE levels. In addition, dogs have a higher number of IgE producing B-cells compared with humans (Ledin *et al*, 2006).

The possibility of reducing IgE levels through vaccination was also investigated by performing an immunization assay on nine Beagle dogs. The vaccine was designed to induce an autoimmune response against IgE and thereby reduce the levels of total IgE. The assumption was that also antigen-specific IgE would be reduced and that it would in that way decrease the allergic symptoms. The dogs were treated with high levels of anti-IgE antibodies, which resulted in a 65% decrease of the mean IgE-levels. This shows that vaccine treatment may be possible as a future therapy of allergic dogs (Ledin *et al*, 2006).

Candidate genes for Canine Atopic Dermatitis

Based on studies in human patients with skin disorders and in murine models of AD, the two genes Kallikrein 7 (KLK7) and Serine Protease Inhibitor Kazal-type 5 precursor (SPINK5) were chosen in the present study as candidate genes for CAD. KLK7 has shown to be involved in important mechanisms in the human skin and may play a part in psoriasis. SPINK5 has shown linkage to AD in humans as well as involvement in another severe skin disease called the Netherton Syndrome (NTS). Both genes are presented below.

The functional role of Kallikrein 7

Desquamation

The stratum corneum is the outermost layer of the epidermis. The corneocytes are cells without nucleus in the stratum corneum representing the last stage of the epidermal differentiation process. Corneocytes consist primarily of keratin filaments surrounded by a cross-linked protein envelope. The stratum corneum has a high mechanical resistance, which is dependent on the cohesion between individual corneocytes. This cohesion is mediated mainly by modified desmosomes. There is a constant production of corneocytes since a fraction of epidermal cells continuously leaves the proliferating basal layer and undergoes differentiation. In order to achieve a constant and well-regulated stratum corneum thickness there is a continuous process of cell shedding at the skin surface. This mechanism is called desquamation which means elimination of stratum corneum cell cohesion in surface cell layers. Proteolysis of intracellular cohesive structures has shown to be of major importance in the series of events that precede desquamation (NCBI´s homepage).

Kallikrein 7

KLK7, also called the stratum corneum chymotryptic enzyme (SCCE), is a serine protease that is specifically expressed in cornifying squamous epithelia (Ekholm & Egelrud, 1999). It is thought to be involved in the desquamation process through proteolysis of intracellular adhesion molecules such as desmoglein. In a study by Ishidia-Yamamoto *et al*, 2005, KLK7 was localized in lamellar granules and more specifically detected in a thin zone at the border between the stratum granulosum and stratum corneum as well as in the stratum corneum. KLK7 may also have other functions unrelated to desquamation i.e. it has shown to be active in catalyzing the creation of active interleukin-1β (IL-1 β) from pro-IL1 β, thus indicating a role in inflammation (Johnson *et al*, 2003). According to Ekholm & Egelrud´s study in 1999 the expression of SCCE in psoriatic skin lesions in humans was found to be consistently increased compared to normal skin. It was, however, not known if this was a secondary effect but it indicated that SCCE may play a part in the aetiology of psoriasis.

The results from a study by Johnson *et al*, 2003, suggest that KLK7 is expressed abnormally in skin where epidermal cell kinetics is disrupted due to inherited or acquired defects.

Serine Protease Inhibitor Kazal-type 5 precursor

The gene SPINK5 is encoding the serine protease inhibitor; Lympho-epithelial Kazal-type related inhibitor (LEKTI). LEKTI consists of 15 potential Kazal-type serine proteinase inhibitory domains (D1-D15) and is expressed in the most differentiated viable layers of stratified epithelial tissue. LEKTI in the epidermis is mainly restricted to the granular layer (Descargues *et al*, 2005), which is the top layer of transcriptionally active keratinocytes, located under the stratum corneum (Galliano *et al*, 2005). It is in the granular layer the crucial biochemical and morphological changes occur during stratum corneum formation (cornification) (Descargues *et al*, 2005).

LEKTI is secreted from the keratinocytes and its possible targets are KLK7 and KLK5 (Kallikrein 5, also called Stratum Corneum Tryptic enzyme (SCTE)). In a study by Ishidia-Yamamoto *et al*, 2005, LEKTI and KLK7 were detected in the lamellar granules but localized separately, with LEKTI detected in the deeper epidermal layers and KLK7 in more superficial layers. The findings from the study suggest that LEKTI is expressed and released earlier than its target proteases (KLK7 and KLK5). Combined, these results show that LEKTI has a role in preventing premature proteolysis of extracellular matrix proteins or cell surface adhesion molecules thus controlling the timing of desquamation.

Netherton Syndrome

SPINK5 has recently been shown to be defective in NTS which is a severe autosomal recessive human skin disorder characterized by congenital ichthyosiform erythroderma, a specific hair shaft defect and atopic manifestations. (Descargues *et al*, 2005) Ichthyotic skin has an abnormal thick stratum corneum and the thickening is a result of hyper-proliferation of the epidermal keratinocytes and/or increased corneocyte adhesion. (Komatsu *et al*, 2002) Individuals affected with NTS have a wide range of allergic manifestations such as AD and elevated IgE levels which are associated with chronic and severe skin inflammation. In NTS, a common feature is the absence of LEKTI expression in the epidermis (Descargues *et al*, 2005).

In NTS patients, the mutations in the SPINK5 gene results in a truncated pro-protein containing fewer inhibitory domains compared with the normal protein. From this follows that KLK7 and KLK5, which are both targets of the inhibitors, are over-activated and this leads to increased degradation of desmoglein 1 (a cell-adhesion molecule crucial for corneocyte adhesion), thus resulting in an over-desquamation of corneocytes (Komatsu *et al*, 2002).

Knock-out mice studies

According to a study by Descargues *et al*, 2005, LEKTI deficiency results in abnormal desmosome cleavage in the upper granular layer, which leads to a defective stratum corneum adhesion and therefore loss of skin barrier function. The study was performed on knock-out mice, $SPINK5^{-/-}$, with the aim to investigate the biological functions of LEKTI. The main conclusion from the study was that LEKTI plays a crucial role in epidermal desquamation, keratinisation, barrier formation and hair morphogenesis. The most notable defect observed in the $SPINK5^{-/-}$ mice was the loss of adherence of stratum corneum, which underlies the severe epidermal fragility.

Linkage to Atopic Dermatitis in humans

In a report by Walley *et al*, 2001, six coding polymorphisms in the human SPINK5 gene were identified. One of these (Glu420-Lys) showed significant association with atopy and AD in two independent sets of families.

Tools in molecular genetic studies

Genetic polymorphisms

Genomes consist of polymorphisms, which are sequences that are found in two or more variants within a population. Single-nucleotide polymorphisms (SNPs) and insertion/deletion polymorphisms (indels) represent the majority of the variation in the genome. SNPs and indels are used to identify loci responsible for a genetic component of a multifactorial phenotypic variation such as disease susceptibility. First, the distribution of SNPs (or indels) must be characterized before an association between a genotypic polymorphism and a certain phenotype can be found. An additional important aspect to consider is the level of haplotype structure due to non-random associations between sites i.e. linkage disequilibrium (LD). A haplotype is a set of adjacent polymorphisms linked together on the same chromosome (Gibson & Muse, 2004).

Only 30-50% of all SNPs have the rare allele present in more than 5% of the individuals within a population. This means that a SNP found in one population might not be useful in mapping experiments of another population. Another relevant aspect of frequency distribution is nucleotide diversity i.e. the average fraction of nucleotides that differ between a pair of alleles chosen at random from a population. Humans have a nucleotide diversity of one SNP every kilobase (kb), which is fairly low. Another aspect is that the amount of variation within a locus differs between different parts of the genome e.g. between introns and exons (Gibson & Muse, 2004).

LD can arise from a variety of sources such as non-random mating, population admixture and epistatic selection (Gibson & Muse, 2004). Dogs have a higher degree of LD within a particular breed compared to humans resulting in longer haplotypes in the dog genome (Sutter *et al*, 2004). This is an effect from the dog's population structure, which has a history of intense inbreeding and a restricted amount of founders within a breed. Due to the long haplotypes, less SNPs are needed in order to evaluate a greater distance of the genome.

Microsatellites

Microsatellites are short simple sequence tandem repeats with a repeat length up to 13 bases (longer repeats are called minisatellites). The most common microsatellites are built up by permutations of di-nucleotide repeats. They are highly abundant in the mammalian genomes and are perhaps the most variable form of DNA sequences known to exist within these genomes. Their polymorphism is caused by length variation of the particular repeat-unit e.g. $(CA)_n$. The frequency distribution of di-nuclotide repeats is: $(CA)_n$>$(AT)_n$>$(GA)_n$>$(GC)_n$. Microsatellite-DNA represents around three percent of the human genome and is distributed at more than one million loci with the following frequency distribution: $(di)_n$, $(mono)_n$, $(tetra)_n$ and least frequent are $(tri)_n$-repeats. (International Human Genome Sequencing Consortium, 2001) Microsatellites occur at an approximate rate of one repeat per 10 kb in eukaryotic genomes, and can be very useful as genetic markers since they are extremely polymorphic with the presence of multiple alleles within a population (Gibson & Muse, 2004).

This study

The primary objective of this project was to search for possible associations between genetic markers, in the vicinity of the chosen dog genes, and the disease phenotype. Bioinformatics was used to find suitable genetic markers near the two candidate genes described above. Three microsatellites in the near vicinity of KLK7 were localized and one intron of SPINK5 was chosen to be sequenced in order to identify informative SNPs.

Materials and Methods

Bioinformatics

The candidate genes chosen for this study were at the time not annotated in the available dog genome sequences but were found in ENSEMBL by aligning the human SPINK5 and KLK7 gene to the dog's genome (ENSEMBL´s homepage).

In dog, SPINK5 is located on chromosome 2 (Cfa2) at location 42,886,822-42,957,059. It consists of 29 exons and has a transcript length of 3,075 base pairs (bp).

In dog, KLK7 is located on chromosome 1 (Cfa1) at location 108,156,349-108,159,472. It consists of 5 exons and its transcript is 774 bp long.

Sample collection

Blood samples from dogs diagnosed with the Willemse´s criteria for CAD, after elimination of different diagnoses such as pyoderma, parasites and CAFR, were collected from 12 veterinary clinics throughout Sweden. The samples were collected between the years 2002 and 2005 and are part of a larger study on naturally occurring canine models of multifactorial diseases where both epidemiological and molecular genetic studies are performed. The multidisciplinary project is performed in collaboration between the Department of Small Animal Clinical Sciences and the Department of Animal Breeding and Genetics, at SLU. The identification of risk factors for CAD will be aided by utilizing unique features of Swedish breed populations. Control animals were chosen at random from the database of the Swedish Kennel Club (SKK) (Nødtvedt *et al*, in press).

Most of the dogs used in this study are listed in the SKK´s register and the dogs' pedigrees were collected from SKK´s database. The parents of the dogs used in this study were defined and compared, leading to the discovery that some test individuals were related to each other.

DNA extraction

Genomic DNA was isolated using a salt extraction method, see Appendix 1. Some of the samples were already extracted by standard methods such as the Phenol-Chlorophorm method and with the E.Z.N.A® Kit; Blood & Body Fluid DNA Spin Protocol. The DNA sample concentrations were measured by the Nanodrop ND-1000 Spectrophotometer and were then diluted with dH_20 to 25 ng/µl.

PCR amplification

Primers were designed using Primer 3 software (Primer3 Software's homepage) and ordered from TAG Copenhagen A/S online (Tag Copenhagen's homepage). The Polymerase Chain Reactions, PCRs, were run with the 2720 Thermal Cycler (Applied Biosystems).

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For each of the three microsatellites one primer pair was designed. The first microsatellite, called Klk71, had the primer sequences; Klk71F: 5´-TCCCACATTAGGCTCTCTGC-3´ and Klk71R: 5´-CACGAATCCAAACCCAATTC-3´. Klk71 consists of a CT-repeat and is located 2.3 kb in front of the gene. The total product size, according to ENSEMBL, was 169 and it consisted of 15 repeats. The second microsatellite, Klk72, had the following primers; Klk72F: 5´-ATGGGGATGGTTCATGATGT-3´ and Klk72R: 5´-TGAGATCCACAGGAGATGGA-3´. Klk72 consists of a TG-repeat and is located 7 kb in front of the gene. It had 16 repeats and a product size of 249 bp according to the sequence in ENSEMBL. The third microsatellite, Klk73, is located 2.3 kb behind the gene and consists of a tetra repeat $(TTTA)_n$. The following primers were designed for the third microsatellite; Klk73F: 5´-CCGGTACTTTTGCCCACTTA-3´ and Klk73R: 5´-TTAGGGTCAAGCCCTGTGTC-3´. The product size of Klk73 was 203 bp and it had 10 repeats.

Instead of labelling each primer pair with dyes for the genotyping laser detection system, a universal fluorescent-labelled M13 (-21) primer was used. All forward primers in each pair were ordered with a M13 (-21) tail at its 5´-end, which had the following sequence; 5´-CACGACGTTGTAAAACGAC-3´. During the first PCR cycles, the forward primers with the M13 (-21) tail are incorporated into the PCR products. The incorporated tails are then targets for the labelled universal M13 (-21) primer. This primer is then included during the remaining cycles which results in a labelled and slightly longer product. The fluorescent dyes used in the PCR were FAM (6-carboxy-fluorescine) for Klk71 and Klk72, and TET (tetrachloro-6-carboxy-fluorescine) for Klk73 (Schuelke, 2000).

After experiencing some problems with optimizing the PCR program, the following mix and program was used successfully for all the microsatellites. 25 ng genomic DNA was amplified with 0.02 µM of the forward primer, 0.2 µM of the reverse primer as well as of the labelled universal M13(-21) primer, 0.5 U of AmpliTaq Gold (Applied Biosystems), 0.2 mM deoxyribonucleoside triphosphate (dNTP), 1x PCR Buffer and 2.0 mM $MgCl_2$ in a final volume of 12.5 µl. The PCR program consisted of a denaturation step of 5 min at 94ºC, followed by 40 cycles of 30 s 94 ºC, 30 s 52 ºC, 25 s 72 ºC and finally an extension step for 15 min in 72 ºC.

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The primers were located in intron five and six and were designed to cover almost all of intron five as well as exon six resulting in a total product size of 1497 bp. The PCR-primer sequences were; Spink5F: 5´-TAGAAATAGTCCTTGCTTTCTG -3´ and Spink5R: 5´-TCCTTCAAAGATTCAAGCCTA – 3´. Also, one forward and one reverse primer were designed for sequencing. These were located in the first part of the fragment with a small

overlap. The sequencing primers were, Spink5seqF: 5´-AATGTGGAGAAGATCCAATG- 3´ and Spink5seqR: 5´-AAGCCTGCTGACATACAGAT- 3´.

For each PCR, after optimization; 50 ng of genomic DNA was amplified with 0.2 µM of each of the PCR-primers, 1.0 U of AmpliTaq Gold (Applied Biosystems), 0.2 mM dNTP, 1x PCR Buffer and 1.5 mM $MgCl_2$ in a final volume of 25 µl. The PCR program consisted of a denaturation step of 5 min at 94ºC followed by 40 cycles of 40 s 94 ºC, 40 s 55 ºC, 3 min 72 ºC and at last an extension step for 5 min in 72 ºC.

Gel extraction and sequencing preparation

To confirm the identity of the fragments, the PCR products were separated on 2% agarose gel (SeaKem®GTG®Agarose and 0.5xTBE).

The SPINK5 fragments were excised from the gel and DNA was extracted with the E.Z.N.A® Kit Gel Extraction Protocol. The fragments were then prepared for sequencing with the kit; DYEnamic ET Dye Terminator Cycle Sequencing Kit for MegaBACE DNA Analysis Systems (Amersham Biosciences). In step 1.2 of the Kit manual the 384-well format was chosen. The mix consisted of a total of 250 ng DNA (for a product size of 1500 kb), 1 µl of primer with a concentration of 5 pmol/µl, 4 µl sequencing reagent premix and water until a total volume of 10 µl was reached. For step 1.4 the program was changed from 1 to 1.5 min for the 60ºC step. In part 2.1.2 30 µl of 95% ethanol was added to each reaction. Before the samples were run on the MegaBACE 10 µl MegaBACE loading solution was added according to step 3.1.

MegaBACE 1000

The MegaBACE 1000 was used for sequencing of the SPINK5 fragments and for genotyping of the KLK7 microsatellites. The buffer plates (one new for each run) consisted of 1x MegaBACE™ LPA Buffer (Amersham Biosciences). The buffer as well as the sample plates were centrifuged 2 min 1000g and the MegaBACE Long Read Matrix (Amersham Biosciences) were centrifuged 3 min 3000g before each run.

For the genotyping of KLK7 microsatellites, each well of the MegaBACE plate consisted of 1 µl of the PCR product and 5 µl of MegaBACE™ ET400-R size standard (diluted 1:20).

For the sequencing of the SPINK5 fragment, the set up of the instrument parameters were according to step 4.3 (MegaBACE 500 and 1000) in the manual used for sequencing preparations.

Analysis

The primary data from the MegaBACE run of the microsatellites were analyzed with the MegaBACE™ Genetic Profiler version 2.2 (Amersham Biosciences). For analyzing of the sequences of the SPINK5 gene the Software; Sequencher™ 3.1.1, was used.

The contingency calculations for the analysis of genetic linkage were preformed with the program CONTING version 2.71 (Utility programs for analysis of genetic linkage, copyright 1988, J. Ott).

Results

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Relationships

Half siblings, full siblings and two cousins were found after the comparison of the dogs' parents.

In the Boxer group, dog 41 and 42, and, dog 28 and 1 were pairs of half siblings (the first dog in each pair was a control dog and the second was a case). Two full sibling pairs were found as well; dog 17 and 49 (both cases) and 16 and 19 (both controls). See Appendix 2, controls are marked green and cases red.

In the WHWT group, dog 3 and 44 (both cases), dog 21 (control) and 25 (case), and, dog 34 and 57 (both controls) were pairs of half siblings. The dogs 45, 47 and 50 (the two first were cases and the last was a control), and, dog 29, 31 and 41 (all controls) were triplets of half siblings. One full sibling pair was found; the control dogs 43 and 15. Dog 28, control, was cousin to the half sibling group of 29, 31 and 41. See Appendix 3.

Allele distribution of microsatellites

For Boxer, three different alleles were found within each of the three microsatellites, except for Klk71 where a fourth allele was found in dog 34. The alleles could be connected into four probable haplotypes (the fourth allele of Klk71 not included). See appendix 2 for the results from the genotyping. In the case where not all three microsatellites worked, the haplotype is marked with a question mark and is included in the calculations within the square brackets. The distribution of alleles and the haplotypes did not show any significant association to case- or control animals, on the other hand they were significantly equally distributed. This trend was also seen when the alleles of the microsatellites were calculated separately. See the analysis of genetic association with contingency calculations of the alleles and haplotypes in Appendix 4.

For WHWT, four different alleles were found within Klk72 and Klk73, and two clear and one uncertain allele were found within Klk71. See Appendix 3 for the results. The alleles found in WHWT could not be connected into any probable haplotypes but calculations could be made concerning the allele frequency in cases and controls. The alleles were equally distributed over cases and controls, thus no significant associations could be seen between a certain allele and the disease phenotype. For the calculations, see Appendix 4.

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Relationships

In the Boxer group there was one pair of half siblings, which consisted of one case (dog 42) and one control animal (dog 41). No relationships were found between any of the WHWTs used in the SNP-analysis of SPINK5.

SNPs and haplotypes

The SNPs found within the sequenced fragment of SPINK5 are shown in Figure 1 below.

Figure 1. The sequenced fragment of SPINK5 and found SNPs.

Sequences marked yellow in the figure are the PCR-primers and sequences marked blue and grey are forward and reverse sequence primers, respectively.

Dog 4 (WHWT) had two insertions; [t/:], marked green, and [g/:], marked blue, and was heterozygote for [c/t] (other dogs were homozygotes for c) at the blue marked position, in the picture above. Dog 20 (WHWT) had three insertions; [gtg/:], marked purple, [g/:] and [t/:], both marked red.

The following SNPs; [a/g] [g/a] [c/a] and [a/g] were found in Boxer (marked grey and bold in the picture). These could be matched together into two haplotypes; AGCA and GAAG. The GAAG haplotype was fixed in WHWT but in Boxer the two haplotypes were found in an about 70:30 ratio. Calculations were made on the distribution of the haplotypes between cases and controls in Boxer, and no associations could be seen between any of the two haplotypes and the disease phenotype. See Appendix 5 for the haplotype distribution and contingency calculations.

Discussion

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The results from the analysis of Klk71 were difficult to interpret and some samples were re-run in order to get more reliable results. In WHWT some peaks looked as if they were one base shorter than the common allele 196 (in WHWT), but in the second and third run the majority of the peaks were estimated to be 196 long. However, two individuals still had peaks which were not easily interpreted even after the re-runs. One of these (47) had a half sibling which was homozygote for the 196 allele, therefore this animal would possibly also carry the 196 allele. The 194 allele found in dog 4 was the one exception from the probably fixed 196 allele in the WHWT. This genotype was fairly definite in its interpretation, since this dog was heterozygote for the 196 and the 194 allele, and no errors could be the cause of this estimation. This dog also had rare alleles in the other loci, which indicates that it is less related to the other dogs. Since the rare alleles are found only in this dog, they were not possible to include in the contingency calculations of allele distribution.

In Boxer, the peaks from the genotyping of dog 41 was difficult to interpret, but was with the help of the probable haplotypes estimated to have the alleles 188/190 in Klk71.

The results from calculations of the allele (WHWT and Boxer) and haplotype (Boxer) distributions indicated with statistical significance that there are no associations between KLK7 and the disease phenotype.

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No haplotypes or SNPs, except individual mutations, were found in WHWT, thus conclusions about the gene's involvement in the disease within this breed can not be drawn. The data shows that the breed probably has a high degree of inbreeding, which explains the lack of polymorphisms in the genome.

In Boxer, the two haplotypes within the SPINK5 gene shows an equal distribution between healthy and sick dogs. However, the haplotype found in this study covers only a small fragment of the genome. This, as well as the fact that there are very few animals included in the study contributes to a low trustworthiness of the results. The results demonstrate breed differences between WHWT and Boxer, showing that the four found SNPs are fixed in WHWT but are polymorphic in Boxer.

Conclusions

The data obtained in this study indicates that the gene KLK7 is not associated with the disease development in Boxer or WHWT. However, if more animals were tested the results would become even more reliable.

Further studies are necessary in order to draw any conclusions concerning the potential involvement of SPINK5 in CAD. SPINK5 should be searched for informative microsatellites in the near vicinity of the gene, so that a similar approach as for the KLK7 gene can be performed. This should lead to more polymorphic markers covering a greater distance. In addition, more animals (cases and controls) must be tested in order to evaluate the SPINK5 gene.

Future research

The Department of Small Animal Clinical Sciences and the Department of Animal Breeding and Genetics, at SLU, are under the process of collecting more samples from CAD-affected as well as healthy dogs of a total of 12 different breeds. These samples, optimally 100 cases and 100 controls within each breed, will be used in a genome-wide association mapping with a panel of 20,000 SNPs. This will hopefully lead to the characterization of a specific region within the genome that is associated with the disease. Candidate genes within this region can then be evaluated further by fine-mapping.

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Appendix 1. DNA extraction protocol

1. Mix 200 µl blood and 200 µl Buffer A in sterile eppendorph tubes. Turn tubes to lysate the cells.
2. Centrifuge for 10 min in 4 ºC in 3000 RPM.
3. Discard the liquid phase carefully and mix the pellet with 300 µl of Buffer A.
4. Redo step 3 until the pellet is light, at least three times.
5. Mix pellet thoroughly with 90 µl of Buffer B.
6. Add 10 µl of Buffer C. The solution should now be viscous.
7. Incubate over night in 50 ºC.
8. Add 27 µl saturated NaCl solution (around 6 M, salt crystals should be seen in the bottom) and shake roughly for 15 seconds.
9. Centrifuge for 15 minutes in 3000 RPM. Move the liquid phase to new tubes.
10. Redo step 9 once.
11. Add 250 µl of cold EtOH. Turn tube.
12. Pick up the DNA with a pipette tip. Transfer the DNA into a new tube with 1xTE or dH_2O (around 30 µl).

Buffer A
0.32 M Sucrose (109.5g)
1 mM TrisHCl ph 7.5 (1 ml, 1 M)
5 mM $MgCl_2$ (5 ml, 1M)
1 % Triton-X (10g)
Add dH_2O until the solution has a volume 1000ml.
Autoclave in 105 ºC
Store in refrigerator

Buffer B
400 mM NaCl (100 ml, 4M)
2 mM EDTA pH 8.0 (10 ml, 0.2M)
10 mM TrisHCl pH 8.0 (10ml, 1M)
Add dH_2O until the solution has a volume 1000ml.
Autoclave in 105 ºC
Store in refrigerator

Buffer C (make fresh the same day)
5 % SDS (200 µl of 10 % solution)
2 µg/µl Proteinase K (100 µl from a stock solution of 8 µg/µl into dH_2O)
100 µl dH_2O

Appendix 2. Results from genotyping of the KLK7 microsatellites in Boxer

Indiv.	Diagnosis	*Klk71* Allele1	Allele2		*Klk72* Allele1	Allele2		*Klk73* Allele1	Allele2	Haplotype1	Haplotype2	
2.6.1	Case	188	188		272	272		224	224	A	A	
2.6.6	Case	190	190		280	280		212	212	B	B	
2.6.7	Case	188	188		272	272		224	224	A	A	
2.6.9	Case	188	196		272	276		224	228	A	C	
2.6.10	Case	190	190		280	280		212	212	B	B	
2.6.11	Case	196	196		276	276		228	228	C	C	
2.6.12	Case	188	196		272	276		224	228	A	C	
2.6.14	Case	188	188		272	280		224	224	A	D	
2.6.17	Case	188	196		272	276		224	228	A	C	
2.6.18	Case	188	196		272	276		224	228	A	C	
2.6.22	Case	188	196									
2.6.29	Case	188	188		272	272				A?	A?	
2.6.31	Case	188	188		272	272		224	224	A	A	
2.6.35	Case	188	196		272	276		224	228	A	C	
2.6.36	Case	190	190		280	280		212	212	B	B	
2.6.37	Case	188	188		272	272				A?	A?	
2.6.38	Case	196	196									
2.6.39	Case				280	280						
2.6.42	Case	190	196		276	280		212	228	B	C	
2.6.46	Case	188	190		272	280				A?	B?	
2.6.49	Case	188	196									
2.6.50	Case	188	188									
2.6.51	Case	188	188									
2.6.52	Case	188	188		272	272				A?	A?	
2.6.53	Case	188	196									25
2.6.15	Control	188	196		272	276		224	228	A	C	
2.6.16	Control	188	196		272	276		224	228	A	C	
2.6.19	Control	188	188		272	280		224	224	A	D	
2.6.24	Control				272	272		224	224	A	A	
2.6.25	Control	188	188		272	272		224	224	A	A	
2.6.26	Control	188	188		272	272		224	224	A	A	
2.6.27	Control	188	196		272	276		224	228	A	C	
2.6.28	Control	188	188		272	272		224	224	A	A	
2.6.32	Control	188	196		272	276		224	228	A	C	
2.6.33	Control	190	196		276	280				B?	C?	
2.6.34	Control	188	198		272	276				A?	?	
2.6.40	Control	188	188		272	272		224	224	A	A	
2.6.41	Control	188?	190?	?	272	280		212	224	A	B	
2.6.43	Control	188	196		272	276		224	228	A	C	
2.6.44	Control	190	196		276	280		212	228	B	C	
2.6.45	Control	188?	196?		272	276				A?	C?	
2.6.47	Control	190	196		276	280		212	228	B	C	
2.6.54	Control	188	188		272	272		224	224	A	A	18

Haplotypes:				
	A:	188	272	224
	B:	190	280	212
	C:	196	276	228
	D:	188	280	224

Half siblings		Full siblings	
2.6.41	2.6.42	2.6.17	2.6.49
2.6.1	2.6.28	2.6.16	2.6.19

Appendix 3. Results from genotyping of the KLK7 microsatellites in WHWT

		Klk71		*Klk72*		*Klk73*		
Indiv.	**Diagnosis**	**Allele1**	**Allele2**	**Allele1**	**Allele2**	**Allele1**	**Allele2**	
3.33.1	Case	196	196	278	280	228	232	
3.33.2	Case	196	196	280	280	228	232	
3.33.3	Case	196	196	278	278	228	228	
3.33.4	Case	**194**	196	274	274	224	228	
3.33.6	Case	196	196	278	278	228	228	
3.33.16	Case	196	196	278	280			
3.33.25	Case	196	196	278	280	232	**236**	
3.33.26	Case	196	196	278	280	228	232	
3.33.27	Case	196	196	280	280			
3.33.30	Case	196	196	278	280	228	228	
3.33.37	Case	195?	195?	280	280	228	228	
3.33.38	Case			278	278	228	228	
3.33.42	Case	196	196	280	280			
3.33.44	Case	196	196	278	280	228	232	
3.33.45	Case			278	278			
3.33.46	Case			280	280	228	232	
3.33.47	Case	196	196	278	280	228	228	
3.33.48	Case	196	196	278	280			
3.33.51	Case			278	280	228	228	
3.33.54	Case			278	280			20
3.33.8	Control	196	196	278	280	228	228	
3.33.13	Control	196	196	280	280	232	232	
3.33.14	Control	196	196	278	280	228	232	
3.33.15	Control			278	280			
3.33.18	Control	196	196	278	280	228	232	
3.33.19	Control	196	196	278	278	228	228	
3.33.20	Control	196	196	278	280	228	232	
3.33.21	Control	196	196	278	280	228	**236**	
3.33.22	Control	196	196	278	280	228	228	
3.33.23	Control	196	196	278	280	228	228	
3.33.24	Control	196	196	278	280	228	228	
3.33.28	Control	196	196	280	280	232	232	
3.33.29	Control	196	196	280	280			
3.33.31	Control			276	280	228	232	
3.33.32	Control	196	196	278	278	224	228	
3.33.33	Control			280	280	228	228	
3.33.34	Control			278	278	228	228	
3.33.39	Control	196	196	280	280	228	232	
3.33.41	Control			280	280	228	232	
3.33.43	Control	196	196	278	280	228	232	
3.33.49	Control	196	196	280	280	228	232	
3.33.50	Control	196	196	278	280	228	232	
3.33.53	Control			278	280	228	228	
3.33.55	Control			280	280	228	228	
3.33.56	Control			278	280			
3.33.57	Control			278	278	228	228	26

Half siblings

3.33.3	3.33.44	
3.33.21	3.33.25	
3.33.47	3.33.50	3.33.45
3.33.34	3.33.57	
3.33.29	3.33.31	3.33.41

Full siblings

3.33.43 3.33.15

Cousins

3.33.28 is cousin to the halfsibling group of 3.33.29, 31 and 41.

Appendix 4. Analysis of genetic associations with contingency calculations

WHWT Klk71

Alleles		*194*	*195*	*196*	*No. Alleles*
Cases (O)		1	2	27	30
Controls (E)		0	0	34	34
Chi square test (2 d.f.):	3.57				
2-sided p-value:	0.16				

WHWT Klk72

Alleles		*274*	*276*	*278*	*280*	*No. Alleles*
Cases (O)		2	0	18	20	40
Controls (E)		0	1	21	30	52
Chi square test (3 d.f.):	3.73					
2-sided p-value:	0.29					

WHWT Klk73

Alleles		*224*	*228*	*232*	*236*	*No. Alleles*
Cases (O)		1	20	6	1	28
Controls (E)		1	31	13	1	46
Chi square test (3 d.f.):	0.61					
2-sided p-value:	0.89					

Boxer Klk71

Alleles		*188*	*190*	*196*	*198*	*No. Alleles*
Cases (O)		27	8	13	0	48
Controls (E)		20	4	9	1	34
Chi square test (3 d.f.):	1.76					
2-sided p-value:	0.62					

Boxer Klk72

Alleles		*272*	*276*	*280*	*No. Alleles*
Cases (O)		19	8	11	38
Controls (E)		21	10	5	36
Chi square test (2 d.f.):	2.52				
2-sided p-value:	0.28				

Boxer Klk73

Alleles		*212*	*224*	*228*	*No. Alleles*
Cases (O)		7	13	8	28
Controls (E)		3	20	7	30
Chi square test (2 d.f.):	3.09				
2-sided p-value:	0.21				

Haplotypes Boxer	***A***	***B***	***C***	**D**	***No. Hapl.***
(in parntesis; all haplotypes incl. individuals with results from only two microsatellites)					
Cases, O	12 (19)	7 (8)		8	1 28 (36)
Controls, E	19 (21)	3 (4)	7 (9)		1 30 (35)
Chi square test (3 d.f.):	3.18 (1.48)				
2-sided p-value:	0.36 (0.69)				

Chi square distribution (0.05 level of sign.)	
3 d.f.	7.81
2 d.f.	5.99

Appendix 5. SNPs found in the intron of the SPINK5 gene

Individual	Diagnosis	Locus 1	Locus 2	Locus 3	Locus 4	Haplotype 1	Haplotype 2
Boxer							
2.6.17	Case	A/A	G/G	C/C	A/A	AGCA	AGCA
2.6.31	Case	A/G	G/A	C/A	A/G	AGCA	GAAG
2.6.35	Case	G/G	A/A	A/A	G/G	GAAG	GAAG
2.6.36	Case	A/A	G/G	C/C	A/A	AGCA	AGCA
2.6.38	Case	A/G	G/A	C/A	A/G	AGCA	GAAG
2.6.42*	Case	A/G	G/A	C/A	A/G	AGCA	GAAG
2.6.32	Control	A/A	G/G	C/C	A/A	AGCA	AGCA
2.6.40	Control	A/A	G/G	C/C	A/A	AGCA	AGCA
2.6.41*	Control	A/A	G/G	C/C	A/A	AGCA	AGCA
2.6.44	Control	A/A	G/G	C/C	A/A	AGCA	AGCA
2.6.47	Control	A/G	G/A	C/A	A/G	AGCA	GAAG
2.6.54	Control	G/G	A/A	A/A	G/G	GAAG	GAAG
WHWT							
3.33.4	Case	G/G	A/A	A/A	G/G	GAAG	GAAG
3.33.16	Case	G/G	A/A	A/A	G/G	GAAG	GAAG
3.33.26	Case	G/G	A/A	A/A	G/G	GAAG	GAAG
3.33.30	Case	G/G	A/A	A/A	G/G	GAAG	GAAG
3.33.6	Case			A/A	G/G	AG	AG
3.33.8	Control	G/G	A/A	A/A	G/G	GAAG	GAAG
3.33.19	Control	G/G	A/A	A/A	G/G	GAAG	GAAG
3.33.20	Control	G/G	A/A	A/A	G/G	GAAG	GAAG
3.33.23	Control	G/G	A/A	A/A	G/G	GAAG	GAAG

Boxer				
Haplotypes	***AGCA***	***GAAG***	***No. Hapl.***	***No. Indiv.***
Cases, O	7	5	12	6
Controls, E	9	3	12	6
Chi square test (1 d.f.):		**0.19**		
2-sided p-value:		**0.665**		

Chi2 distribution (0.05 level of sign.)		
1 d.f.	3.84	

** Half siblings*