โครงการ: ปัจจัยทางพันธุกรรมและอุบัติการณ์ของโรคสะบ้าเคลื่อนในสุนัขพันธุ์เล็ก

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บทคัดย่อ

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ชื่อโครงการ: ปัจจัยทางพันธุกรรมและอุบัติการณ์ของโรคสะบ้าเคลื่อนในสุนัขพันธุ์เล็ก

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การศึกษาทางพันธุกรรมและอุบัติการณ์ของโรคสะบ้าเคลื่อนในสุนัขที่มารับการรักษา ที่โรงพยาบาลสัตวเล็ก คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ตั้งแต่ปี พ.ศ. 2549 จนถึงปี พ.ศ. 2551 ทำการตรวจทางพันธุกรรมเพื่อหาความสัมพันธ์ของโรคสะบ้าเคลื่อนในสุนัขพันธุ์ปอมเมอเรเนียจำนวน 16 ครอก กับ microsatellite markers ที่อยู่ใกล้กับคอลลาเจนโปรตีนต่างๆ จำนวน 5 ตัว ได้แก่ COL6A1, COL6A3, COL9A1, COL9A2 และ COL9A3 ค่า lod score ที่ได้จากการวิเคราะห์ผลด้วยวิธี linkage analysis แบบ recessive inheritance model คือ 0.19, 0.05, 0.29, 0.53 และ 0.42 และแบบ dominant inheritance model คือ –0.75, -0.99, -1.01, 0.24 และ –0.61 ตามลำดับ ค่าที่ได้จากการคำนวณด้วย nonparametric linkage analysis โดยโปรแกรม Genehunter ได้ค่า NPL score สูงสุดคือ 1.56 ของ COL9A2 โดยค่า p-value เท่ากับ 0.07 และค่า lod score สูงสุดจากการคำนวณของสุนัขทั้ง 16 ครอก คือ 0.85 และ 1.27 สำหรับ recessive และ dominant model ตามลำดับ ค่า lod score ที่ได้จากการศึกษาครั้งนี้มีค่าน้อย แสดงให้เห็นว่าไม่มีความสัมพันธ์ระหว่าง markers ดังกล่าวกับโรคสะบ้าเคลื่อนในสุนัขพันธุ์ปอมเมอเรเนีย

คำสำคัญ สุนัข สะบ้าเคลื่อน พันธุกรรม

Abstract

Project code: MRG5080124

Project title: Genetic factor and incidence of patellar luxation in small breed dogs

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Project period: 1 December 2006-30 November 2008

Genetic factor and incidence of patellar luxation (PL) were screened in dogs present at the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University during 2006-2008. DNA screenings were collected from PL littermates and their parents. We found the high incidence of PL in the Pomeranian 16 famillies, and 5 polymorphic DNA markers situated closely to the COL6A1, COL6A3, COL9A1, COL9A2, and COL9A3 genes were analyzed. Under a recessive inheritance model with incomplete penetrance of 90% for the genotype at risk and 10% of phenocopies, the lod scores for the COL6A1, COL6A3, COL9A1, COL9A2, and COL9A3 were 0.19, 0.05, 0.29, 0.53, and 0.42, respectively. The lod scores with a dominant model and the same percentages of penetrance and phenocopies were –0.75, -0.99, -1.01, 0.24, and –0.61, respectively. From sib-pair analysis with Genehunter software, none of the markers analyzed showed a high nonparametric linkage score. The highest NPL score of 1.56 was obtained for COL9A2 with a *p* value of 0.07. The maximum lod score obtained from 16 families was 0.85 and 1.27 for the recessive model and the dominant model, respectively. The low lod scores found in this study indicated that there is no linkage of the COL6A1, COL6A3, COL9A1, COL9A2, and COL9A3 collagen genes with patellar luxation in the Pomeranian.

Keywords: Dog, Patellar luxation, Genetic

Genetic factor and incidence of patellar luxation in small breed dogs

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Abstract

Genetic factor and incidence of patellar luxation (PL) were screened in dogs present at the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University during 2006-2008. DNA screenings were collected from PL littermates and their parents. We found the high incidence of PL in the Pomeranian 16 famillies, and 5 polymorphic DNA markers situated closely to the COL6A1, COL6A3, COL9A1, COL9A2, and COL9A3 genes were analyzed. Under a recessive inheritance model with incomplete penetrance of 90% for the genotype at risk and 10% of phenocopies, the lod scores for the COL6A1, COL6A3, COL9A1, COL9A2, and COL9A3 were 0.19, 0.05, 0.29, 0.53, and 0.42, respectively. The lod scores with a dominant model and the same percentages of penetrance and phenocopies were –0.75, -0.99, -1.01, 0.24, and –0.61, respectively. From sib-pair analysis with Genehunter software, none of the markers analyzed showed a high nonparametric linkage score. The highest NPL score of 1.56 was obtained for COL9A2 with a *p* value of 0.07. The maximum lod score obtained from 16 families was 0.85 and 1.27 for the recessive model and the dominant model, respectively. The low lod scores found in this study indicated that there is no linkage of the COL6A1, COL6A3, COL9A1, COL9A2, and COL9A3 collagen genes with patellar luxation in the Pomeranian.

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1. Introduction

Patellar luxation is one of the most common orthopaedic disorders found in small breed dogs which can result in the development of degenerative joint disease, pain, and lameness.[1-4] In recent years, the incidence among large dogs appears to be increasing.[3,5-6] This disorder is considered

developmental resulting from multiple anatomical abnormalities of the pelvic limbs. The pathogenesis of patellar luxation has been extensively reviewed but still remains unclear.[4,7-9] However, a heritable basis for the disease has been suggested, which is supported by the predisposition of certain breeds including Miniature and Toy poodles, Yorkshire Terriers, Pomeranian, Pekingese, Chihuahuas, and Boston Terriers.[1,10] Lateral patellar luxation (LPL) is uncommon and is reported to occur more often in large-breed dogs.[11]; however, medial patellar luxation is more frequently recognized in dogs of all sizes.[5] There appears to be a sex predilection with the risk of medial patellar luxation for females being one and half times that for males.[5]

In Thailand, there is high incidence of patellar luxation (PL) in the newly born dogs of small breeds. 87% and 13% of dogs are medial and lateral PL, respectively.[12] During the last decade, knowledge on SIB pair analysis and whole genome scanning has been used for the development of DNA-screening methods for various abnormalities in some breeds of dogs.[13] A uniformly clinical screening demonstrated the affected litters and families. Since high incidence of PL in Pomeranians, it is interesting to study molecular genetic of this disease. DNA samples from dogs with PL mainly Pomeranian were collected for SIB pair analysis.

1.1 Functional Anatomy

Limb dysfunction caused by medial or lateral patella luxation is one of the most common problems seen in hindlimb lameness. To choose the most appropriate treatment method, the surgeon must know the normal anatomy, function, and interrelationships of the hip joint, femur, and tibia. In the normal angle of the femoral neck-femoral shaft axis in the frontal projection is approximately 135 to 145 degrees. Coxa valga is an increase in the angle of the femoral neck-femoral shaft axis, whereas caxa vara is a decrease in that angle. Anteversion of the femoral head and neck is an external rotation of the proximal femur in relation to the distal femur. Retroversion is the opposite: an internal rotation of the femoral head relative to the distal femur. The normal anteversion angle in puppies is near 0 degrees and increases to approximately 27 degrees in the adult. Distally, the angle of the transcondylar-femoral shaft axis in the frontal projection is approximately 93 degrees. The femoral trochlear sulcus is the wide articular groove on the cranial surface of the femur that articulates with the patella. The groove is bounded medially and laterally by prominent trochlear ridges that aid in maintaining stability of the patella. The trochlear groove is normally in alignment with the quadriceps mechanism, patellar ligament, and tibial tuberosity. This anatomical alignment is necessary for stability of the stifle joint and efficiency of the extensor mechanism.

The patella is an ossified portion of the quadriceps tendon and plays an important role in the extensor mechanism of the stifle. The power for extensor mechanism comes from the four heads of the quadriceps femoris muscle group. Three of these including vastus lateralis, vastus intermedius, and vastus medialis originate from the proximal femur and the fourth, rectus femoris, originates from the ilium. These four converge to insert on the patella and then continue on to form the strong patellar

ligament which inserts on the tibial tuberosity. The patella rides in the trochlear groove of the femur, as well as its articular surface is convex and corresponds to the concave shape of the trochlear groove. The trochlear groove is formed by the lateral and medial trochlear ridges which project from the cranial surface of supracondylar region of the distal femur, and thus cradle the patella. The vastus lateralis and vastus medialis insert onto the patella by well-developed fibrocartilagenous plates called the parapatellar cartilages. These articulate with the trochlear ridges and increase the surface area of contact, thus spreading the force of the quadriceps muscle. The vastus medialis counteracts the lateral pull of the vastus intermedius and lateralis on the patella as the stifle is extended, so that the patella remains in its normal position. The patella has a number of important functions including maintains even tension as the stifle is extended, increases the mechanical leverage applied by the quadriceps group, and decreases friction between the quadriceps and condyles. As the stifle moves from flexion to extension, the patella follows a medial to lateral sinus arc. At the end of extension the patella is found buttressed against the lateral trochlear ridge.[13]

The tibial tuberosity is located cranial and distal to the tibial condyles. Its location and prominence are important for the mechanical advantage of the extensor mechanism. The alignment of the quadriceps, patella, trochlea, patellar tendon, and tibial tuberosity must be normal for proper function. The trochlear ridges, the quadriceps group and the joint capsule and retinaculum all help to stabilize the patella in the trochlear groove during an extension excursion. A normal balance and direction of the forces applied by these structures is essential for normal joint stability. In particular, abnormal alignment of the extensor mechanism results in abnormal mechanics and joint instability, which in turn places abnormal stresses on the ligaments and menisci of the stifle and may directly result in osteoarthritis. In the growing dog, the consequences of these abnormal forces are compounded by their effects on the growing cartilages (growth plate and articular cartilage) of the distal femur and proximal tibia.[14]

1.2 Canine genetic diseases

1.2.1 Dogs as a study model for inherited diseases

The dog (Canis lupus familiaris) was originally domesticated by man about 15,000 years ago. Selective breeding regarding to behavior and morphological traits over the years has led to formation of numerous different breeds. In the present day, each breed consists of a closed population of purebred dogs, which all have certified pedigree registration. For a dog to become recognized as a member of a specified breed, its parents need to be registered to that breed. Genetic variation within a breed is limited by the small number of original founders. Inbreeding can cause loss of genetic diversity and bottlenecks in population size and popular sire effects, and can have a negative impact on the genetic variation within a breed. Along with the canine characteristics on which man has selected purposely by selective breeding, undesired mutations have been co-selected for unknowingly. Nowadays, approximately 338 breeds are recognized by Federation Cynologique Internationale and

155 breeds by the American Kennel Club. Among these breeds a large variation exists with respect to weight, size, proportion, shape, coat, behavior and diseases. Each breed seems to have its own set of hereditary diseases, and the NCBI database Online Mendelian Inheritance in Animals[15] currently lists approximately 480 different disease phenotypes in the dog. More than one genetic cause might exist for a condition between breeds. The most frequently occurring diseases incluse cancers, epilepsy, hip dysplasia, thyroid disease, allergies, bloat, heart disease, autoimmune disease, progressive retinal atrophy and cataracts.[16] Many of these are also of major health concern to humans.

The dog is an interesting animal model for researches in human disorders, as this species has advantages over rodents. Firstly, the life span, body size, and physiology of dogs are more similar to human than those of rodents.[17] At the DNA level, the coding sequences of dogs and humans show an overall similarity to each other more than to the coding sequence of mice, indicating a closer evolutionary relationship of the two species. Secondly, canine diseases resemble in general more closely to human diseases than to rodent diseases.[16] Moreover, the diseases in dogs are naturally occurring as opposed to the diseases studied in rodents which are often induced.[17] Thirdly, dogs live in the same environment as their human owner. Finally, veterinary medical care for dogs resembles human medicine to a high degree in both diagnostics and therapy. Advantages of studying a hereditary disease in a canine population in comparison to a human one include a shorter generation time in dogs, a higher number of offspring per pregnancy, and the availability of pedigree registration. Disadvantages of use of dogs as an animal model include the long gestation and the high maintenance costs of breeding programs. Canine diseases can be used as a model for human diseases to learn to understand the molecular biology of common cellular processes and disorders. This is illustrated by the studies of incidence and genetic aspects of patellar luxation in small breed dogs in Thailand.

1.2.2 Phenotyping

The phenotype of an individual is the total of observable characteristics resulting from the interaction of its genotype and the environment. The basis of genetic studies of hereditary diseases lies in the correct determination of the phenotype, that is, a reliable identification of patients and unaffected individuals. This requires a clear description of the disease and its diagnostic criteria, and preferably a protocol based clinical work-up. When it comes to the choice of diagnostic tools to be used, the risk for the animal and owner and costs need to be considered. Also the choice of the owner needs to be respected in case of privately owned dogs. In some cases ethics should be considered, for instance when one is interested in determining the phenotype of animals without any disease symptoms.

When exposing seemingly healthy family members to invasive medical procedures in order to determine their phenotype and/or obtain a DNA sample to be able to study a hereditary disease in the family, the burden for the individual (potential risks, invasiveness, stress) has to be weighed against the burden of the disease in the breed. Advantage of diagnostics in animals without symptoms is that

this could lead to early identification of affected individuals, which implies an early start of therapy and an owner knowing what to expect in the future. Potential problem in the process of phenotyping can be caused by diseases with phenocopies and by a late onset. Phenocopies are individuals with identical clinical pictures (phenotype), simulating the effect of a certain genotype, but with an environmental background or caused by another gene. Existence of phenocopies in a sample set could interfere in a genetic study and should therefore always be considered during the phenotyping process. In case of a disease with a late onset, classification of animals as unaffected might be hard to impossible if indications of disease are absent. To overcome this in the statistical analysis, such individuals can be classified with an unknown phenotype, or a model with age dependent liability classes can be used. Alternatively, the analysis can be set up on an affecteds only basis, in which only the genotypes of affected individuals are compared.

1.3 Sampling

1.3.1 Genetic model

Knowledge of the genetic model of a disease is important in genetic studies. A genetic model consists of a hypothesis about the mode of inheritance of a disease, the penetrance of each genotype and the gene frequency. The segregation pattern of a disease can only be reliably established by studying the phenotypes of complete families. Besides pedigree studies, breeding trials could also help to establish the mode of inheritance of a disease. In case a sample set consists of isolated, independent cases or if it is hard to establish the genetic model of a disease, a model free or non-parametric analysis of the samples can be used alternatively. Examples of these include affected sib pair analyses and association studies.

1.3.2 Methodology to study the genetics of PL

Linkage and association analysis can be used to identify genes that cause disease. Linkage analysis compares segregation of DNA marker data with segregation of the phenotype in families, whereas association analysis determines the frequency of marker alleles in groups of affected and unaffected dogs. Both linkage analysis and association studies can be performed by means of either preselected candidate gene approach or by way of a whole-genome scan.

Collagen is the collective term of the filamentous protein component of the connective tissue. Collagen gene defects are at the basis of a large number of diseases in humans, and these disorders are often caused by inherited mutations in genes encoding collagen protein.[18] To analyze collagen genes as candidates for involvement in these disorders in dogs, closely situated polymorphic DNA markers form a useful tool.

Microsatellites are tandem repeats of di-, tri-, or tetranucleotides, which are found dispersed along genomes of vertebrates.[19] A natural variation in repeat number occurs frequently at a microsatellite locus, resulting in variation of DNA sequence length and polymorphism. These length variants or alleles are stably transmitted to the offspring, and the markers can be used to study genetic

linkage or association with phenotypes such as an inherited disorder. A disease that has been linked to a microsatellite marker is, at least in part, caused by DNA mutations in the proximity of that marker.

2. Material and Methods

2.1 Patients

Patellar luxation was screened in dogs present at the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University during 2006-2008. Blood samples for DNA screening were collected from PL littermates and their parents. 95 DNA samples were collected from Pomeranian dogs that had patellar luxations. 59 affected healthy dogs were selected from 16 families. These families were analyzed for co-segregation of the phenotype with 5 polymorphic DNA markers situated closely to the COL6A1, COL6A3, COL9A1, COL9A2, and COL9A3 genes (table 1).[20] Mlink was used to calculate the lod score (scaled log likelihood ratio) for the linkage of the phenotype with each of the markers. The results were statistically analyzed with Genehunter software. Linkage of the gene with PL is determined when there is more than 50% of the genome sharing with the sibling pairs. The results were statistically evaluated with Genehunter software.

Single Nucleotide Polymorphisms (SNPs) have become available in large numbers to the canine genetic community from the genome sequencing projects. A total of 608,222 putative canine SNPs were detected in the Poodle. Analysis of the genome sequence data of the Boxer itself and comparison of the draft sequence of the Boxer to the Poodle and to nine additional breeds in 6% of the genome has revealed a comprehensive set of SNPs useful in all dog breeds. Studies in which candidate genes were searched for SNPs indicated that, in addition to the many SNPs that have become available by sequencing of the dog genome, many more canine SNPs exit. Even when only detected in a single breed, a high percentage of SNPs found in one breed can be expected to be polymorphic in other breeds, as well. So, we used SNPs in order to specify regions of interest in order to find genes associated with PL in Pomeranians which has the advantage of being model free.

2.2 DNA isolation from blood

2.2.1 Collecting the nuclei and denaturation of proteins

Blood sample was collected and mixed with EDTA in a 15-ml tube. The buffy coat fraction was obtained by centrifuging the whole blood sample for 15 minutes at 3,000 rpm and 4°C, then collected in the labeled 50 ml-tube, mixed quietly with 5 ml of 1x Erythrocytenlysis buffer (RBC) by hand shake (not vortex), and then put on ice for at least 30 minutes. The sample was centrifuged for 10 minutes at 2,500 rpm and 4°C; then the supernatant was carefully removed. The pellet of white blood nuclei was resuspended in 20 ml of 1x RBC, shaken firmly by hand to resuspend the pellet,

centrifuged for 10 minutes at 2,500 rpm and 4°C; then the supernatant was removed. The pellet was resuspended in 0.6 ml of salt extraction-lysis buffer per 1 ml of the buffy coat, shaken firmly by hand, added 22.5 µl of pronase per 1 ml of the buffy coat and 64 µl of 10% SDS per 1 ml of the buffy coat (denaturation of proteins), mixed carefully by swirling the tube, and then left over night at 37°C in the incubator or for the weekend at room temperature.

2.2.2 Collection of the DNA

After the solution is clear, it was added 0.2 ml of 6M NaCl per 1 ml of the buffy coat and shaken firmly by hand for 30 seconds. The unclear solution was added with 100-200 µl more pronase and incubated for another 2 hours at 37°C. The supernatant was decanted in a 15-ml tube with 2 ml of absolute ethanol and the tube was gently turned upside down twice. There will appear a DNA suspension in the fluid. The DNA was picked up with a sterile plastic graft eye, washed with 1 ml of 70% ethanol (to remove the salt), and dissolved in a labeled microcentrifuge tube with 0.1 ml of Tris-EDTA per 1 ml of the buffy coat, and then left for 10 minutes in a 65°C water bath (inactivation of the DNAse). DNA was preserved at -20°C.

Several collagen genes were localized by BLASTN searches of the completed dog genome DNA sequence at www.ncbi.nlm.nih.gov/genomes. The human reference cDNA sequence for collagen type VI alpha 1 (COL6A1) (NM_001848), COL6A3 (NM_004369), COL9A1 (NM_078485), COL9A2 (NM_001852) and COL9A3 (NM_001853) were used for the localizations. The resulting hits displayed high similarity to the cDNA and could be identified as the coding regions of the corresponding collagen genes. The coding DNA sequences of the human and dog genes were approximately 90% identical, and the dog genes were in each case the only genes that displayed this high level of similarity. The localization of these reconstructed genes on the canine genome is shown in Table 1. Microsatellite repeats that were possibly polymorphic and informative for linkage analysis were selected from the genomic DNA sequences in the vicinity of the genes with Tandem Repeat Finder software.[21]

Oligonucleotides (primers) for amplification of the microsatellite markers were designed with Primer3 software (Rozen and Skaletsky, 2000) and the 5' end of the forward primers were tailed with the M13 forward sequencing primer (GTTTTCCCAGTCACGAC).

The PCR reactions of 15 µl contained 25 ng of genomic DNA, 1 µM M13 tail forward primer, 10 µM reverse primer, 10 µM M13 forward sequencing primer labeled at the 5' end with HEX fluorophore (Eurogentec, Seraing, Belgium), 1×PCR gold buffer, 2.5 mM $MgCl_2$, 1mM dNTPs, and 0.3 U Amplitaq Gold (Applied Biosystems, Foster City, CA, USA).

Thermal cycling was carried out in the GeneAmp 9700 (Applied Biosystems, Foster City, CA, USA) with the following program: 5 minutes at 95°C, followed by 10 cycles of 30 seconds at 95°C, 15 seconds at the annealing temperature (table 1), 30 seconds at 72°C, then another 25 cycles of 30

seconds at 92°C, 15 seconds at the annealing temperature, and 30 seconds at 72°C. The program was completed with 10 minutes at 72°C.

The PCR reaction were diluted 10 to 30 folds with H_2O. 2 µl of the dilution was mixed with 10 µl formamide and 0.2 µl GS500 LIZ or TAMRA size standard (Applied Biosystems). The products were analyzed after capillary electrophoresis and automatically detected using the Genetic Analyzer 3100 (Applied Biosystems).The DNA products were classified by size with Genescan Analysis version 3.7 software (Applied Biosystems) and alleles were assigned.

3. Results

From 2006 to 2008, 236 dogs with PL were found in various breeds including 175 Pomeranian (74.15 %), 24 Poodle (10.17 %), 14 Chihuahua (5.93 %), 3 Pug (1.27 %), 3 Bull dog (1.27%), 2 Yorkshire Terrier (0.85 %), 2 Maltese Terrier (0.85 %), 2 Miniature Pincher (0.85 %), 1 Shih Tzu (0.42 %), 1 Terrier (0.42%), 1 Cocker Spanial (0.42 %), 1 Labrador Retriever (0.42 %), and 7 mixed breed dogs (2.97 %). Among 175 Pomeranians, medial patellar luxation was diagnosed in 314 joints (96.62 %), while lateral PL was present in 11 joints (3.38 %). 150 dogs (85.71 %) were bilaterally affected while 25 dogs (14.29 %) were unilaterally affected. Incidences of breeds, sexes, and grades of PL in dogs were shown in Table 2.

95 DNA samples were collected from Pomeranian dogs that had patellar luxations. From this sample population, 59 affected and healthy dogs were selected from 16 families of Pomeranians. These families were analyzed for co-segregation of the phenotype with 5 polymorphic DNA markers situated closely to the collagen genes COL6A1, COL6A3, COL9A1, COL9A2, and COL9A3.

Mlink was used to calculate the lod score for linkage of the phenotype with each of the markers (Table 3). Under a recessive inheritance model with incomplete penetrance of 90% for the genotype at risk and 10% of phenocopies, the lod scores for the COL6A1, COL6A3, COL9A1, COL9A2, and COL9A3 were 0.19, 0.05, 0.29, 0.53, and 0.42, respectively. The lod score with a dominant model and the same percentages of penetrance and phenocopies was –0.75, -0.99, -1.01, 0.24, and –0.61, respectively. From sib-pair analysis with Genehunter software, none of the markers analyzed showed a high nonparametric linkage score. (Table 4)

The highest NPL score of 1.56 was obtained for COL9A2 with a p value of 0.07. The maximum lod score obtained from 16 families was 0.85 and 1.27 for the recessive model and the dominant model, respectively. These maximum lod scores are too low to pinpoint the definite linkage. This is caused by the large number of affected dogs in the pedigrees. Analysis should be switched to genome-wide association testing, which has the additional advantage of being model free.

4. Discussion

This study selected the gene coding for collagen type 6 alpha-1 (COL6A1), COL6A3, COL9A1, COL9A2, and COL9A3 as candidate genes because all of these are involved in human bone disorders such as patellar luxation, hyperextension syndrome, and multiple epiphyseal dysplasia. The low lod scores found in this study indicated that there is no linkage of the COL6A1, COL6A3, COL9A1, COL9A2, and COL9A3 collagen genes with patellar luxation in Pomeranians. The linkage is justified at the lod scores of 3 or higher.[22] The large number of affected dogs in the pedigrees might have some effects on the low scores. According to a number of genes relating and unrelating to each canine disorder, it will be helpful if there is a screening procedure for ruling out the genes that are less associated with that disorder.

The linkage analysis is based on comparison of genotypes of pairs of affected siblings. The principle is as follows: two littermates share 50% of their genome at random. However, littermates which are affected by an inherited disorder will share the causative genes. Therefore, on average, pairs of affected littermates will share more than the expected 50% of the regions with disease genes, irrespective of a dominant or recessive mode of inheritance. Sibling pair analysis can be applied when a large number of pairs are available and the mode of inheritance is not known. It can also circumvent the problem of heterogeneity, i.e. when different genes can cause the disorder independently. We do not anticipate heterogeneity, however, because a dog breed can be regarded as genetically homogeneous isolate. With the sib-pair approach there are no problems with reduced penetrance because only affected dogs are included in the initial analysis.

Single Nucleotide Polymorphisms (SNPs) have become available in large numbers to the canine genetic community from the genome sequencing projects. A total of 608,222 putative canine SNPs were detected in the Poodle. Analysis of the genome sequence data of the Boxer itself and comparison of the draft sequence of the Boxer to the Poodle and to nine additional breeds in 6% of the genome has revealed a comprehensive set of SNPs useful in all dog breeds. Studies in which candidate genes were searched for SNPs indicated that, in addition to the many SNPs that have become available by sequencing of the dog genome, many more canine SNPs exit. Even when only detected in a single breed, a high percentage of SNPs found in one breed can be expected to be polymorphic in other breeds, as well. So, SNPs might be one of the supporting tools for screening the genes that are most likely associated with patellar luxation in dogs.

Knowledge of the gene will help a further study of the aetiology of PL. The expression and function of the gene will be studied at biochemical and histological levels. The expression of the defective gene will be followed by in situ hybridization studies. The distribution of the protein product in normal and affected tissues will be determined by immunohistochemical techniques.

Acknowledgements

This project was support by the Thailand research fund. We would like to thank P.A.J. Leegwater and H.A.W. Hazewinkel from the department of clinical sciences of companion animals, Faculty of Veterinary Medicine, Utrecht University, The Netherlands. We would like to thank J. Temwichitr from the department of companion animals clinical sciences, Faculty of Veterinary Medicine, Kasetsart University, Thailand.

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Table 1 Genomic locations of candidate genes for patellar luxation and microsatellite markers.

Gene	CFA[a]	Mb[b]	O[c]	Microsatellite PCR oligonucleotides	T[d]	Range (bp)	#[e]	Human phenotype of gene mutation
COL6A1	31	41.6	F	aactgcttactcattgaagg	60	204-216	4	Patellar luxation, hyperextension syndrome
			R	acttgcaaccatcacattac				
COL6A3	25	51	F	gtcacttggcaccaggttag	60	330-335	4	Patellar luxation, hyperextension syndrome
			R	gtcaccagatgcagctcaga				
COL9A1	12	36.0	F	gattaactccaggcagaatc	60	306-312	4	Multiple epiphyseal dysplasia
			R	cctaggagtgactctctgct				
COL9A2	15	5.0	F	ctaccagcaggcaactccaa	60	353-357	3	Multiple epiphyseal dysplasia
			R	caggatgccaacgagaacaa				
COL9A3	24	49.4	F	gctccagagttcaggctcag	55	213-223	4	Multiple epiphyseal dysplasia
			R	aggaggtcggagatggagat				

[a]CFA = *Canis familiaris* chromosome number; [b]Position in mega base pairs of chromosome DNA sequence; [c]Orientation of oligonucleotides; F = forward primer, R = reverse primer; [d]Annealing temperature in ºC; [e]Number of alleles.

Table 2 Incidences of breeds, sexes, and grades of patellar luxations in dogs present at the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University during 2006 and 2008.

	Number of dogs	Number of joints affected with PL		
		Total joint	Medial	Lateral
Breeds				
Pomeranian	175	325	314	11
Poodle	24	42	42	0
Chihuahua	14	24	24	0
Yorkshire Terrier	2	4	4	0
Maltese Terrier	2	3	3	0
Miniature Pincher	2	4	4	0
Pug	3	4	3	1
Bull dog	3	5	5	0
Shih Tzu	1	2	2	0
Terrier	1	2	2	0
Cocker Spanial	1	2	0	2
Labardor Retriever	1	2	2	0
Mixed	7	14	7	7
Sex				
Female	147			
Male	89			
Total	236			
Grading (1 to 4)				
Grade 1		116	113	3
Grade 2		188	183	5
Grade 3		94	85	9
Grade 4		35	31	4

Table 3 Two-point lod score of collagen genes in Pomeranian.

	COL6A1	COL6A3	COL9A1	COL9A2	COL9A3
Recessive model	0.19	0.05	0.29	0.53	0.42
Dominant model	-0.75	-0.99	-1.01	0.24	-0.61

Table 4 Results of the sib-pair and nonparametric linkage (NPL) analysis.

Gene	NPL score	p-value	Information content
COL6A1	0.25	0.59	0.49
COL6A3	1.09	0.89	0.58
COL9A1	0.64	0.74	0.65
COL9A2	1.56	0.07	0.60
COL9A3	0.49	0.31	0.24

Fig 1 Pedigrees of Pomeranian dogs. Circles and squares represent female and male dogs, respectively. Filled symbols indicate PL dogs.

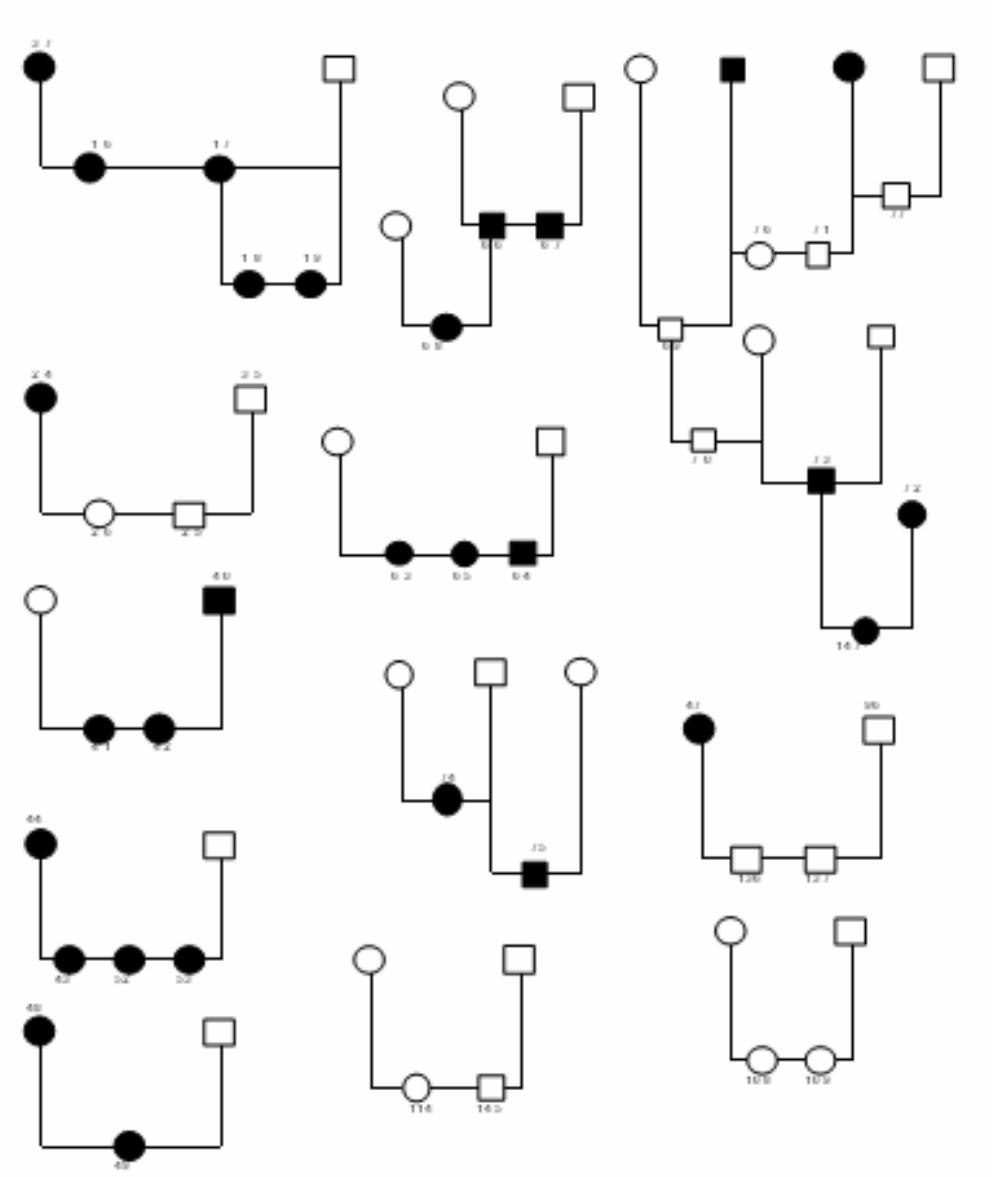


Fig 2 Pedigrees of Poodle dogs. Circles and squares represent female and male dogs, respectively. Filled symbols indicate PL dogs.

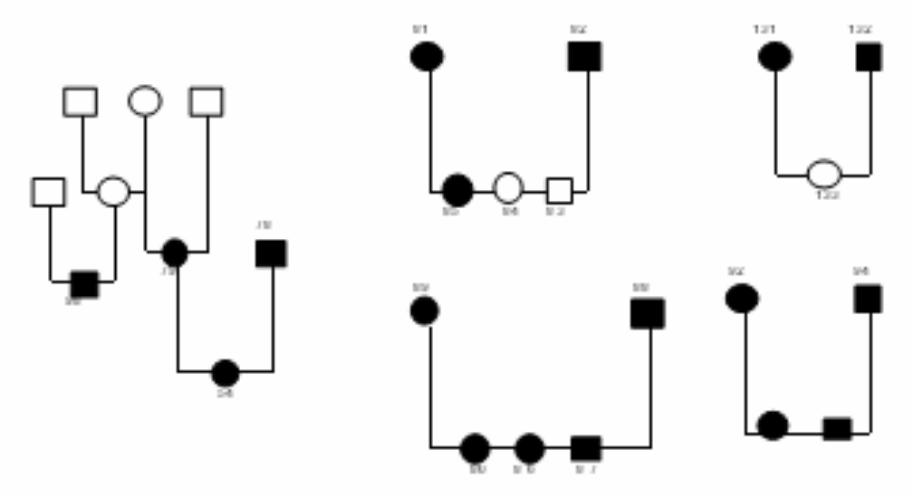


Fig 3 Pedigrees of Chihuahua dogs. Circles and squares represent female and male dogs, respectively. Filled symbols indicate PL dogs.

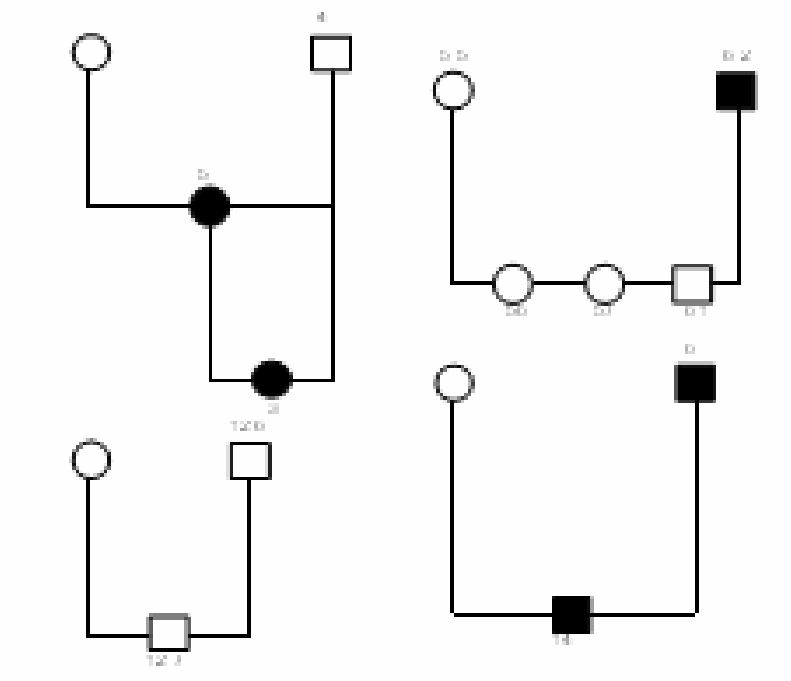
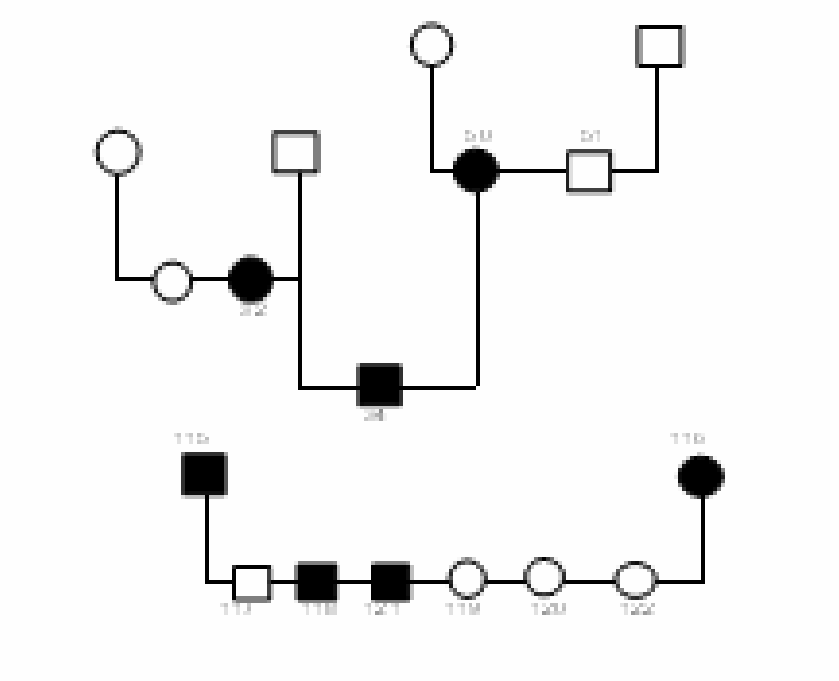


Fig 4 Genome wide linkage analysismof patellar luxation in Pomeranian dogs. For each chromosome (CFA number indicated in the center top) the chi square and permutation p-value is shown.



Output ที่ได้จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. เรื่องสั้นตีพิมพ์ในวารสารรวบรวมผลงานทางวิชาการภาคบรรยาย ของงานประชุมวิชาการระดับนานาชาติ The 4th World Congress on Regenerative Medicine ในหัวข้อเรื่อง Wangdee, C., Soontornvipart, K., Kalpravidh, M., Sarikaputi, M., Brahmasa, A. 2009. Incidence and Genetic aspects of patellar luxation in small breed dogs in Thailand. The 4th World Congress on Regenerative Medicine. 12-14 Mar 2009. Bangkok, Thailand.

2. การนำผลงานวิจัยไปใช้ประโยชน์

เชิงพาณิชย์ เกิดแนวทางในการจัดการการผสมพันธุ์สุนัข เพื่อเพาะพันธุ์สุนัขในเชิงพาณิชย์

เชิงนโยบาย เกิดแนวทางในการจัดเก็บข้อมูลสุนัขในฟาร์มที่ทำการเพาะพันธุ์สุนัขจำหน่าย เพื่อใช้ในการสืบค้นปประวัติของสุนัขแต่ละตัว รวมทั้งควรมีการตรวจโรคที่จำเป็นด้วย เพื่อเป็นข้อมูลในภายภาคหน้า

เชิงสาธารณะ การนำผลการวิจัยไปขยายผลในการศึกษาทางโรคอื่นๆ ที่มีการถ่ายทอดทางพันธุกรรม

เชิงวิชาการ ทำให้มีแนวทางการทำวิจัยในแนวลึกต่อเนื่อง เพื่อประโยชน์แก่วิชาชีพสัตวแพทย์

3. อื่น ๆ

นำเสนอผลงานวิจัยในรูปแบบ Poster ที่ การประชุมวิชาการระดับนานาชาติ The 4th World Congress on Regenerative Medicine กรุงเทพมหานคร ประเทศไทย ระหว่างวันที่ 12-14 มีนาคม 2009 จำนวน 1 เรื่อง

ภาคผนวก

Incidence and genetic aspects of patellar luxation in small breed dogs in Thailand

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Introduction

The dog (Canis lupus familiaris) was originally domesticated by humans about 15,000 years ago. Selective breeding regarding to behavior and morphological traits over the years has led to formation of numerous different breeds. In the present day, each breed consists of a closed population of purebred dogs, which all have certified pedigree registration. For a dog to become recognized as a member of a specified breed, its parents need to be registered to that breed. Genetic variation within a breed is limited by the small number of original founders. Inbreeding can cause loss of genetic diversity and bottlenecks in population size and popular sire effects, and can have a negative impact on the genetic variation within the breed. Along with the canine characteristics on which man has selected purposely by selective breeding, undesired mutations have been co-selected for unknowingly. Nowadays, approximately 338 breeds are recognized by Federation Cynologique Internationale and 155 breeds by the American Kennel Club. Among these breeds a large variation exists with respect to weight, size, proportion, shape, coat, behavior, and diseases. Each breed seems to have its own set of hereditary diseases, and the NCBI database Online Mendelian Inheritance in Animals[1] currently lists approximately 480 different disease phenotypes in the dog. More than one genetic cause might exist for a condition between breeds. The most frequently occurring diseases include cancers, epilepsy, hip dysplasia, thyroid disease, allergies, bloat, heart disease, autoimmune disease, progressive retinal atrophy and cataracts.[2] Many of these are also of major health concern to humans.

Patellar luxation (PL) is one of the most common orthopaedic disorders found in small breed dogs which can result in the development of degenerative joint disease, pain, and lameness.[3-6] In recent years, the incidence among large dogs appears to be increasing.[5,7-8] This disorder is considered developmental and is resulted from multiple anatomical abnormalities of the pelvic limbs. The pathogenesis of patellar luxation has been extensively reviewed but still remains unclear.[6,9-11] However, a heritable basis for the disease has been suggested, which is supported by the predisposition of certain breeds including Miniature and Toy poodles, Yorkshire Terriers, Pomeranian, Pekingese, Chihuahuas, and Boston Terriers.[3,12] Lateral patellar luxation is uncommon and is reported to occur more often in large-breed dogs.[13] Medial patellar luxation is

more frequently recognized in dogs of all sizes and appears to be a sex predilection with the risk for females at one and half times that for males.[7]

During the last decade, knowledge on SIB pair analysis and whole genome scanning has been used for the development of DNA-screening methods for various abnormalities in some breeds of dogs.[14] A uniformly clinical screening demonstrated the affected litters and families. In Thailand, there is high incidence of patellar luxation in the newly born dogs of small breeds. 87% and 13% of dogs are medial and lateral PL, respectively.[15] Since high incidence of PL in small breed dogs, it is interesting to study molecular genetic of this disorder. Finding of this study will be beneficial to the selective breeding of dogs. Defects in collagen are at the basis of a large number of diseases in humans, and these disorders are often caused by inherited mutations in genes encoding collagen proteins.[16] Among a number of collagen genes, collagen type 6 alpha-1 (COL6A1), COL6A3, COL9A1, COL9A2, and COL9A3 are involved in human bone disorders such as patellar luxation, hyperextension syndrome, and multiple epiphyseal dysplasia.[17] The purpose of this study was to find if there is the involvement of these genes with patellar luxation in dogs.

Materials and methods

Patients

Patellar luxation was screened in dogs present at the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University during 2006-2008. Blood samples for DNA screening were collected from PL littermates and their parents. Because of a high incidence of PL in the Pomeranian, DNA samples collected from 59 affected healthy Pomeranian dogs from 16 families were analyzed for co-segregation of the phenotype with 5 polymorphic DNA markers situated closely to the COL6A1, COL6A3, COL9A1, COL9A2, and COL9A3 genes (table 1).[17] Mlink was used to calculate the lod score for the linkage of the phenotype with each of the markers. The results were statistically analyzed with Genehunter software. Linkage of the gene with PL is determined when there is more than 50% of the genome sharing with the sibling pairs. The results were statistically evaluated with Genehunter software.

Table 1. Genomic locations of candidate genes for patellar luxation and microsatellite markers.

Gene	CFA[a]	Mb[b]	O[c]	Microsatellite PCR oligonucleotides	T[d]	Range (bp)	#[e]	Human phenotype of gene mutation
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COL6A3	25	51	F	gtcacttggcaccaggttag	60	330-335	4	Patellar luxation, hyperextension syndrome
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COL9A1	12	36.0	F	gattaactccaggcagaatc	60	306-312	4	Multiple epiphyseal dysplasia
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COL9A2	15	5.0	F	ctaccagcaggcaactccaa	60	353-357	3	Multiple epiphyseal dysplasia
			R	caggatgccaacgagaacaa				
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[a]CFA = *Canis familiaris* chromosome number; [b]Position in mega base pairs of chromosome DNA sequence; [c]Orientation of oligonucleotides; F = forward primer, R = reverse primer; [d]Annealing temperature in [o]C; [e]Number of alleles.

DNA isolation from blood

Collection of the nuclei and denaturation of proteins

Blood sample was collected and mixed with EDTA in a 15-ml tube. The buffy coat fraction was obtained by centrifuging the whole blood sample for 15 minutes at 3,000 rpm and 4°C, then collected in the labeled 50 ml-tube, mixed quietly with 5 ml of 1x Erythrocytenlysis buffer (RBC) by hand shake (not vortex), and then put on ice for at least 30 minutes. The sample was centrifuged for 10 minutes at 2,500 rpm and 4°C; then the supernatant was carefully

removed. The pellet of white blood nuclei was resuspended in 20 ml of 1x RBC, shaken firmly by hand to resuspend the pellet, centrifuged for 10 minutes at 2,500 rpm and 4°C; then the supernatant was removed. The pellet was resuspended in 0.6 ml of salt extraction-lysis buffer per 1 ml of the buffy coat, shaken firmly by hand, added 22.5 µl of pronase per 1 ml of the buffy coat and 64 µl of 10% SDS per 1 ml of the buffy coat (denaturation of proteins), mixed carefully by swirling the tube, and then left over night at 37°C in the incubator or for the weekend at room temperature.

***C**ollection of the DNA*

After the solution is clear, it was added 0.2 ml of 6M NaCl per 1 ml of the buffy coat and shaken firmly by hand for 30 seconds. The unclear solution was added with 100-200 µl more pronase and incubated for another 2 hours at 37°C. The supernatant was decanted in a 15-ml tube with 2 ml of absolute ethanol and the tube was gently turned upside down twice. There will appear a DNA suspension in the fluid. The DNA was picked up with a sterile plastic graft eye, washed with 1 ml of 70% ethanol (to remove the salt), and dissolved in a labeled microcentrifuge tube with 0.1 ml of Tris-EDTA per 1 ml of the buffy coat, and then left for 10 minutes in a 65°C water bath (inactivation of the DNAse). DNA was preserved at -20°C.

Several collagen genes were localized by BLASTN searches of the completed dog genome DNA sequence at www.ncbi.nlm.nih.gov/genomes. The human reference cDNA sequence for COL6A1 (NM_001848), COL6A3 (NM_004369), COL9A1 (NM_078485), COL9A2 (NM_001852) and COL9A3 (NM_001853) were used for the localizations. The resulting hits displayed high similarity to the cDNA and could be identified as the coding regions of the corresponding collagen genes. The coding DNA sequences of the human and dog genes were approximately 90% identical, and the dog genes were in each case the only genes that displayed this high level of similarity. The localization of these reconstructed genes on the canine genome is shown in Table 1. Microsatellite repeats that were possibly polymorphic and informative for linkage analysis were selected from the genomic DNA sequences in the vicinity of the genes with Tandem Repeat Finder software.[18]

Oligonucleotides (primers) for amplification of the microsatellite markers were designed with Primer3 software (Rozen and Skaletsky, 2000) and the 5' end of the forward primers were tailed with the M13 forward sequencing primer (GTTTTCCCAGTCACGAC).

The PCR reactions of 15 µl contained 25 ng of genomic DNA, 1 µM M13 tail forward primer, 10 µM reverse primer, 10 µM M13 forward sequencing primer labeled at the 5' end with HEX fluorophore (Eurogentec, Seraing, Belgium), 1×PCR gold buffer, 2.5 mM $MgCl_2$, 1mM dNTPs, and 0.3 U Amplitaq Gold (Applied Biosystems, Foster City, CA, USA).

Thermal cycling was carried out in the GeneAmp 9700 (Applied Biosystems, Foster City, CA, USA) with the following program: 5 minutes at 95°C, followed by 10 cycles of 30 seconds at 95°C, 15 seconds at the annealing temperature (table 1), 30 seconds at 72°C, then another 25 cycles of 30 seconds at 92°C, 15 seconds at the annealing temperature, and 30 seconds at 72°C. The program was completed with 10 minutes at 72°C.

The PCR reaction were diluted 10 to 30 folds with H_2O. 2 µl of the dilution was mixed with 10 µl formamide and 0.2 µl GS500 LIZ or TAMRA size standard (Applied Biosystems). The products were analyzed after capillary electrophoresis and automatically detected using the Genetic Analyzer 3100 (Applied Biosystems).The DNA products were classified by size with Genescan Analysis version 3.7 software (Applied Biosystems) and alleles were assigned.

Results

Incidences of breeds, sexes, and grades of PL in dogs were shown in table 2. Among 236 dogs with PL found in this study, there were 175 Pomeranians (74.15 %), 24 Poodles (10.17 %), 14 Chihuahuas (5.93 %), 3 Pugs (1.27 %), 3 Bull dogs (1.27%), 2 Yorkshire Terriers (0.85 %), 2 Maltese Terriers (0.85 %), 2 Miniature Pinchers (0.85 %), 1 Shih Tzu (0.42 %), 1 Terrier (0.42%), 1 Cocker Spanial (0.42 %), 1 Labrador Retriever (0.42 %), and 7 mixed breed dogs (2.97 %). In 175 Pomeranians, medial PL was diagnosed in 314 joints (96.62 %) while lateral PL was observed in 11 joints (3.38 %). Bilateral and unilateral luxations were found in 150 (85.71 %) and 25 dogs (14.29 %), respectively.

The lod scores (scaled log likelihood ratio) for linkage of the phenotype with each of the markers are shown in table 3. Under a recessive inheritance model with incomplete penetrance of 90% for the genotype at risk and 10% of phenocopies, the lod scores for the COL6A1, COL6A3, COL9A1, COL9A2, and COL9A3 were 0.19, 0.05, 0.29, 0.53, and 0.42, respectively. The lod scores with a dominant model and the same percentages of penetrance and phenocopies were –0.75, -0.99, -1.01, 0.24, and –0.61, respectively. From sib-pair analysis with Genehunter software, none of the markers analyzed showed a high nonparametric linkage score (Table 4).

Table 2. Incidences of breeds, sexes, and grades of patellar luxations in dogs present at the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University during 2006 and 2008.

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Terrier	1	2	2	0
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COL9A2	1.56	0.07	0.60
COL9A3	0.49	0.31	0.24

The highest NPL score of 1.56 was obtained for COL9A2 with a *p* value of 0.07. The maximum lod score obtained from 16 families was 0.85 and 1.27 for the recessive model and the dominant model, respectively. These maximum lod scores are too low to pinpoint the definite linkage.

Discussion

This study selected the gene coding for collagen type 6 alpha-1 (COL6A1), COL6A3, COL9A1, COL9A2, and COL9A3 as candidate genes because all of these are involved in human bone disorders such as patellar luxation, hyperextension syndrome, and multiple epiphyseal dysplasia. The low lod scores found in this study indicated that there is no linkage of the COL6A1, COL6A3, COL9A1, COL9A2, and COL9A3 collagen genes with patellar luxation in the Pomeranian. The linkage is justified at the lod scores of 3 or higher.[19] The large number of affected dogs in the pedigrees might have some effects on the low scores. According to a number of genes relating and unrelating to each canine disorder, it will be helpful if there is a screening procedure for ruling out the genes that are less associated with that disorder.

Single Nucleotide Polymorphisms (SNPs) have become available in large numbers to the canine genetic community from the genome sequencing projects. A total of 608,222 putative

canine SNPs were detected in the Poodle. Analysis of the genome sequence data of the Boxer itself and comparison of the draft sequence of the Boxer to the Poodle and to nine additional breeds in 6% of the genome has revealed a comprehensive set of SNPs useful in all dog breeds. Studies in which candidate genes were searched for SNPs indicated that, in addition to the many SNPs that have become available by sequencing of the dog genome, many more canine SNPs exit. Even when only detected in a single breed, a high percentage of SNPs found in one breed can be expected to be polymorphic in other breeds, as well. So, SNPs might be one of the supporting tools for screening the genes that are most likely associated with patellar luxation in dogs.

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Incidence and genetic aspects of patellar luxation in Pomeranian dogs in Thailand

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In Thailand, there is a high incidence of patellar luxation (PL) in the newly born dogs of small breeds. 87% and 13% of dogs are medial and lateral PL, respectively.[1] During the last decade, knowledge on SIB pair analysis and whole genome scanning has been used for the development of DNA-screening methods for various abnormalities in some breeds of dogs.[2] A uniformly clinical screening demonstrated the affected litters and families. Since high incidence of PL in Pomeranians, it is interesting in studying molecular genetic of this disease.

Objective

To assess the linkage of 5 collagen genes namely COL6A1, COL6A3, COL9A1, COL9A2, and COL9A3 with patellar luxation (PL) in Pomeranian dogs.

Materials and methods

Blood samples for DNA screening were collected from PL littermates and their parents. 95 DNA samples were collected from Pomeranian dogs that had patellar luxations. 59 affected healthy dogs were selected from 16 families of Pomeranians. These families were analyzed for co-segregation of the phenotype with 5 polymorphic DNA markers situated closely to the COL6A1, COL6A3, COL9A1, COL9A2, and COL9A3 genes (table 1).[3] Mlink was used to calculate the lod score for the linkage of the phenotype with each of the markers. The results were statistically analyzed with Genehunter software. Linkage of the gene with PL is determined when there is more than 50% of the genome sharing with the sibling pairs.

Table 1. Genomic locations of candidate genes for patellar luxation and microsatellite markers

Gene	CFA[a]	Mb[b]	O[c]	Microsatellite PCR oligonucleotides	T[d]	Range (bp)	#[e]	Human phenotype of gene mutation
COL6A1	31	41.6	F	aactgcttactcattgaagg	60	204-216	4	Patellar luxation, hyperextension syndrome
			R	acttgcaaccatcacattac				
COL6A3	25	51	F	gtcacttggcaccaggttag	60	330-335	4	Patellar luxation, hyperextension syndrome
			R	gtcaccagatgcagctcaga				
COL9A1	12	36.0	F	gattaactccaggcagaatc	60	306-312	4	Multiple epiphyseal dysplasia
			R	cctaggagtgactctctgct				
COL9A2	15	5.0	F	ctaccagcaggcaactccaa	60	353-357	3	Multiple epiphyseal dysplasia
			R	caggatgccaacgagaacaa				
COL9A3	24	49.4	F	gctccagagttcaggctcag	55	213-223	4	Multiple epiphyseal dysplasia
			R	aggaggtcggagatggagat				

[a]CFA = *Canis familiaris* chromosome number; [b]Position in mega base pairs of chromosome DNA sequence; [c]Orientation of oligonucleotides; F = forward primer, R = reverse primer; [d]Annealingtemperature in [o]C; [e]Number of alleles.

Results

Incidences of breeds, sexes, and grades of PL in dogs present at the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University during 2006 and 2008 were shown in table 2. In a recessive inheritance model with incomplete penetrance of 90% for the genotype at risk and 10% of phenocopies, the lod scores for the COL6A1, COL6A3, COL9A1, COL9A2, and COL9A3 were 0.19, 0.05, 0.29, 0.53, and 0.42, respectively. The lod scores for the COL6A1, COL6A3, COL9A1, COL9A2, and COL9A3 in a dominant model of the same percentages of penetrance and phenocopies as in a recessive inheritance model were –0.75, -0.99, -1.01, 0.24, and –0.61, respectively (table 3). From sib-pair analysis with Genehunter software, none of the markers analyzed showed a high nonparametric linkage score (table 3).

Table 2. Incidences of breeds, sexes, and grades of patellar luxations in dogs during 2006 and 2008

	Number of dogs	Number of joints affected with PL		
		Total joint	Medial	Lateral
Breeds				
Pomeranian	175	325	314	11
Poodle	24	42	42	0
Chihuahua	14	24	24	0
Yorkshire Terrier	2	4	4	0
Maltese Terrier	2	3	3	0
Miniature Pincher	2	4	4	0
Pug	3	4	3	1
Bull dog	3	5	5	0
Shih Tzu	1	2	2	0
Terrier	1	2	2	0
Cocker Spanial	1	2	0	2
Labardor Retriever	1	2	2	0
Mixed	7	14	7	7
Sex				
Female	147			
Male	89			
Total	236			
Grading (1 to 4)				
Grade 1		116	113	3
Grade 2		188	183	5
Grade 3		94	85	9
Grade 4		35	31	4

Table 3. Results of the sib-pair and nonparametric linkage (NPL) analysis

Gene	NPL score	*p*-value	Information content
COL6A1	0.2447	0.5877	0.4854
COL6A3	1.0875	0.8894	0.5817
COL9A1	0.6379	0.7424	0.6492
COL9A2	1.5625	0.0657	0.6003
COL9A3	0.4937	0.3053	0.2415

Discussion

This study found no linkage of the COL6A1, COL6A3, COL9A1, COL9A2, and COL9A3 collagen genes with patellar luxation in pomeranian dogs. The large number of affected dogs in the pedigrees might cause the low lod scores. SNPs (Single Nucleotide Polymorphisms) with the advantage of its model free might be used at the specific regions of interest in order to find the genes associated with patellar luxation in Pomeranians.

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