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REVIEW ARTICLE

GENETIC BACKGROUND OF CHONDRODYSPLASIA IN DOMESTIC DOG (*CANIS LUPUS FAMILIARIS*) – *IN SILICO* ANALYSIS

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Abstract. Chondrodysplasia is a type of genetic skeletal disorder associated with abnormalities in the development of cartilage tissues. Affected dogs suffer from disproportionate short-limbed dwarfism and/or hyperplasia of the skull bones. Visible symptoms occur only during growth period. Early studies demonstrated that chondrodysplasia in domestic dog is an autosomal recessive disease. Therefore, the only way to detect carriers in the population without performing test matings is a genetic test. Current knowledge of the genetic background of chondrodysplasia is limited to Norwegian Elkhound and Karelian Bear Dog. In case of other breeds, for example Labrador Retriever, known research only excludes participation of certain genes in the origin of the disease. Therefore, the authors of this article decided to develop diagnostic genetic tests that will detect carriers on the basis of previously identified mutations, causing chondrodysplasia.

Key words: chondrodysplasia, domestic dog, Labrador Retriever, COL11A2, Norwegian Elkhound, ITGA10

Skeletal dysplasias is a heterogenous group of disorders associated with bone and cartilage development. Impaired osteosis and chondrification lead to abnormalities in cartilage growth and dwarfism of limbs of various degrees [Young et al. 2008]. Both in humans and canines some skeletal disorders are known to be of genetic origin. Chondrodysplasia, also known as chondrodystrophia, is a genetic

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disorder of epiphysial cartilage growth. It was first described in 1952 in French Bulldog and Dachshund breeds [Hansen 1952].

The main phenomenon leading to this disorder is a messy proliferation and maturation of cartilage cells, resulting in abnormalities in cartilagenous osteosis. Consequently, bones in limbs and spine may be shortened (dwarfism), together with excessive growth of skull bones (so-called "pug head"). In some breeds these features are considered desirable and have been deliberately bred for in breeds such as Bulldog, Basset Hound and Pekingese. Due to the fact that puppies do not display any pathological symptoms, for these appear only during later development [Bengtsson et al. 2005, Mosallanejed et al. 2007], the disease is difficult to diagnose at an early age. Although rentgenography can be performed just after birth, its results cannot be reliably assessed before a puppy is 5 to 12 weeks old. Chondrodysplasia has been found in many dog breeds, e.g. Alaskan Malamute [Padgett et al. 1969, Subden et al. 1972], Norwegian Elkhound Grey [Bingel and Sande 1982, Kyöstilä et al. 2013], Karelian Bear Dog [Kyöstilä et al. 2013], Miniature Poodle [Gardner 1959, Riser et al. 1980], Labrador Retriever [Carrig et al. 1977], Deerhound [Breur et al. 1989], English Pointer [Lavelle 1984], Pyrenean Mountain Dog [Bingel and Sande 1994], Irish Red Setter [Hanseen et al. 1998], and also in sheep, goats, cattle and humans. It has also been found in German Shepherd [Roberg 1979, Mosallanejed et al. 2007]. In the latter case the affected specimen came from healthy parents and its biochemical and morphological blood parameters were within normal, also in case of calcium and phosphorus serum concentration [Mosallanejed et al. 2007].

Current strategies in combating chondrodysplasia are limited to excluding affected (homozygous recessive) specimens from breeding. However, taking in account the autosomal recessive mode of its inheritance, this strategy remains ineffective and does not provide effective eradication of the disease. Additionally, test matings are performed to detect heterozygotes (carriers) – when any affected puppies are born, both parents are excluded from breeding. This strategy is also far from being effective, because the propability of producing homozygous (affected) puppies from two heterozygotes does not exceed 25% and thus selection is slow. This strategy has also other disadvantages, as elimination of all heterozygous specimens (which can produce perfectly healthy offspring when mated to "clear" partners) results in narrowing the gene pool. Moreover, some desired genes can be accidentally lost along with the unwanted ones.

Research have proven the autosomal recessive pattern of inheritance in the following breeds: Alaskan Malamute [Subden et al. 1972], Pyrenean Mountain Dog [Bingel and Sande 1994], Norwegian Elkhound Grey [Kyöstilä et al. 2013] and Irish Setter [Hanseen et al. 1998]. In some other breeds the disease is supposed

to have genetic background, yet its inheritance pattern remains unknown [Kyöstilä et al. 2013].

Currently we do not have effective prevention of inhibiting the spread of disease in the population, as well as effective methods for treatment. Dogs affected by mild to moderate form of chondrodysplasia usually do not feel pain because of changes in the development of cartilage tissue, but these individuals should always be under the care of a veterinarian. Treatment is directed primarily on the symptoms associated with chondrodysplasia and is mainly aimed at pain relief and reducing the discomfort of the animal.

Retrotransposition as a cause of chondrodysplasia

Retrotransposition, i.e. the process of duplication of genetic material and switching it to a new location, is a frequent source of new sequences that appear in the evolution of genomes. Many of the genes, resulting from this process, are inactive pseudogenes lacking promoters. Research by Parker et al. [2009] demonstrated that in result of retrotranposition the *fgf4* retrogene appeared in the canine genome and it is probably linked to chondrodysplasia in domestic dogs. The *fgf4* itself is a type 4 fibroblasts growth factor inducing expression of SPRY genes. These genes, via ubiquitine, disrupt degradation of FGF receptors, including FGFR3.

Its expression is limited only to the period of the growth of the long bones, yet – due to the presence of the *fgf4* gene also in its active retrogene form – it is icreased and thus may give rise to the chondrodyplastic phenotype found in humans and mice, among others [Parker et al. 2009].

Genes not linked to chondrodysplasia

Research by Young et al. [2006], Young and Bannasch [2008] showed that neither the COL10A1 (type 10 colagen encoding gene) nor RMRP and SHOX are responsible for the development of canine chondrodysplasia. These genes had been earlier considered as candidates, for they are involved in different skeletal dysplasias in humans. However, detailed studies excluded participation of the mutations within encoding sequences and promoter regions of these genes in the development of canine chondrodysplasia.

Norwegian Elkhound (Grey) and Karelian Bear Dog

The Norwegian Elkhound (Grey) is one of the breeds classified in FCI Group 5 – Spitz and Primitive Types. It is a hunting dog, popular in the Nordic co-

untries. Chondrodysplasia was described in this breed in the 1980s [Bingel and Sande 1982]. However, it was not before 2013 when its genetic backround was established on the request of the Finnish breeders, worried about high percentage of affected specimens in the population. Based on genome wide association study (GWAS), the postulated candidate gene was found and identified as ITGA10 [Kyöstilä et al. 2013].

Provided the ITGA10 gene sequence is normal, its product is $\alpha 10$ integrin subunit; together with $\beta 1$ chain it forms a heterodimeric receptor on the chondrocyte surface [Kyöstilä et al. 2013, Lundgren-Åkerlund and Aszòdi 2014]. $\alpha 10\beta 1$ integrin is the most often occuring of all colagen-binding integrins in the connective tissue and a key mediator of interactions between the cell and intercellular matrix, indispensable for the normal development of cartilage [Lundgren-Åkerlund and Aszòdi 2014]. Chondrodysplasia is caused by substitution of 2083. nucleotide in the encoding sequence (XM_845262.1) (cytosine (C) into thymine (T) – c.2083C>T (Fig. 1). This mutation leads to the presence of UGA codon, terminating translation process (p.Arg695*). As a result, termination of the synthetized protein occurs earlier and the resulting shorter product does not meet its proper function [Kyöstilä et al. 2013]. As 2083. nucleotide is also 8460. nucleotide of the ITGA10 gene sequence (CanFam3.1.:17:58,697,792:58,712,394) (Fig. 2), it is possible to design PCR primers enabling amplification of the mutation site.

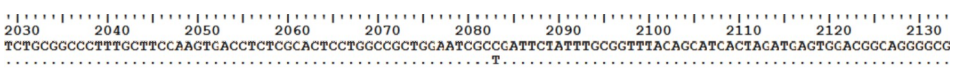

Fig. 1. Encoding sequence comparison of the wild and the mutated allele of the ITGA10 (XM_845262.1) gene in Norwegian Elkhounds Grey – BioEdit program (v.7.2.5)

Rys. 1. Porównanie sekwencji kodującej allelu dzikiego z sekwencją kodującą allelu zmutowanego genu ITGA10 (XM_845262.1) u psów rasy elkund szary dokonano za pomocą programu BioEdit (v.7.2.5)

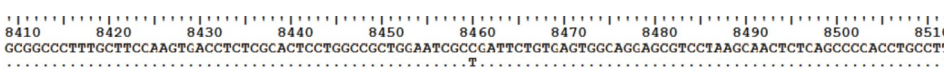

Fig. 2. Encoding sequence comparison of the wild and the mutated allele of the ITGA10 (CanFam3.1:17:58,697,792:58,712,394) gene in Norwegian Elkhounds Grey – BioEdit program (v.7.2.5)

Rys. 2. Porównanie sekwencji allelu dzikiego z sekwencją allelu zmutowanego genu ITGA10 (CanFam3.1:17:58,697,792:58,712,394) u psów rasy elkund szary dokonano za pomocą programu BioEdit (v.7.2.5)

Previous research demonstrate that deletion of $\alpha 10$ subunit in mice leads to delayed growth of long bones that can described as a mild form of chondrody-

splasia, characterized by changed chondrocyte shape and lowered proliferation, disorders in column organisation of growth plate and lower density of colagen fibres in cartillage matrix [Bengtsson et al. 2005].

This mutation occurs also in chondrodysplastic Karelian Bear Dogs and most probably was introduced into their population many years ago in result of deliberate crosses with Norwegian Elkhounds [Kyöstilä et al. 2013].

Commonly used methods of molecular genetics enable fast and reliable detection of carriers of the mutated ITGA10 gene in canine population (Ensembl: ENSCAFG00000011346.4). On the basis of *in silico* analysis, the authors of this paper propose the following diagnostic scheme, which allows detection of heterozygous specimens.

Due to inability to employ the fastest and easiest method using restriction fragment length polymorphism, instead we propose allele specific PCR (AS-PCR). This method can diagnose any known mutation with the use of primers specific for the sequence that includes the locus of the mutation.

First, two independent amplification reactions are run with two different primers pairs. To amplify the wild allele (PCR "A") primers of the sequence: forward – 5'ATCGC**C**GATTCTGTGAGTG3', reverse – 5'ACAGGTGACATTCCCTACGC3' can be used, while the mutated allele (PCR "B") can be amplified with the use of identical primer reverse and primer forward of 5'GCTGGAATCGC**T**GATTCTGT3' sequence. The resulted PCR products are of 225 and 231 pz length, respectively, and this difference is too small to distinguish bands in 1.5% agarose gel, yet in case of AS-PCR method it is not necessary. Carrying out two independent amplications and visualisation of their products in agarose gel allows for clear identification of the mutation. Healthy specimens (double wild allele) show positive result only when PCR reaction is carried out with primers complementary to the wild allele (PCR "A"), affected ones (double mutated allele) – with primers complementary to the mutated allele (PCR "B"), whereas in case of heterozygous specimens (carriers) the band results only from both reactions (Fig. 3).

Labrador Retriever

Genetic background of chondrodysplasia in this breed remains yet unknown. Research by Frischknecht et al. [2013] found the reason for mild disproportionate dwarfism, which is considered a mild form of chondrodysplasia [Frischknecht et al. 2013]. According to their results, the condition is caused by a specific causative mutation in the COL11A2 (type 11 colagen encoding gene), more precisely – substitution of 143. nucleotide in XM_538855.2 encoding sequence (guanine to cytosine) – c.143G>C (Fig. 4), which leads to substitution of aminoacids (arginine to proline) – p.Arg48Pro. As this nucleotide is also 3425. nucleotide in the

COL11A2 gene sequence (CanFam3.1.:2,626,222:2,656,298) (Fig. 5), it is possible to design PCR primers enabling amplification of the mutation site.

Specimen Osobnik	Homozygote, 2x wild allele Homozygota, 2x allel dziki		Heterozygote Heterozygota		Homozygote, 2x mutated allele Homozygota, 2x allel zmutowany	
Reaction type Typ reakcji	PCR "A"	PCR "B"	PCR "A"	PCR "B"	PCR "A"	PCR "B"
Result Wynik		no band brak prążka			no band brak prążka	

Fig. 3. Electrophoretic patterns of different genotypes (dominant homozygote, recessive homozygote, heterozygote) as obtained by the authors after in silico analysis

Rys. 3. Wzór wyniku rozdziału elektroforetycznego homozygoty dominującej, recesywnej oraz heterozygoty po analizie in silico przeprowadzonej przez autorów

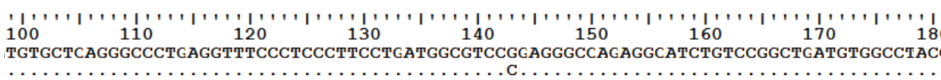

Fig. 4. Encoding sequence comparison of the wild and the mutated allele of the COL11A2 (XM_538855.2) gene in Labrador Retrievers – BioEdit program (v.7.2.5)

Rys. 4. Porównanie sekwencji allelu dzikiego z sekwencją allelu zmutowanego genu COL11A2 (XM_538855.2) u psów rasy labrador retriever dokonano za pomocą programu BioEdit (v.7.2.5)

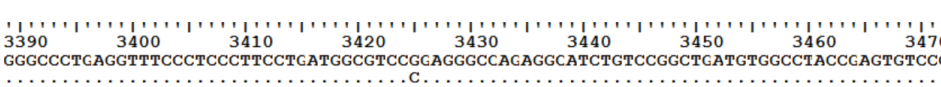

Fig. 5. Encoding sequence comparison of the wild and the mutated allele of the (CanFam3.1: 2,626,222:2,656,298) gene in Labrador Retrievers – BioEdit program (v.7.2.5)

Rys. 5. Porównanie sekwencji allelu dzikiego z sekwencją allelu zmutowanego genu COL11A2 (CanFam3.1: 2,626,222:2,656,298) u psów rasy labrador retriever dokonano za pomocą programu BioEdit (v.7.2.5)

Studies *in silico* demonstrate high tolerance of organisms to changes in protein primary structure. However, type 11 colagen is an important molecule and any alterations in its structure may impair its functions. Type 11 colagen, together with type 2 and 9 molecules, is indispensable for the proper differentiation and spacial organisation of growth plate chondrocytes [Frischknecht et al. 2013]. Studies based on microsatellitar marker polymorphism excluded any participation of other colagen encoding genes (COL9A1, COL9A2, COL9A3, COMP, MATN3, COL2A1, COL11A1) in the development of chondrodysplasia in Labrador Retrievers [Smit et al. 2011].

Commonly used methods of molecular genetics allows for fast and reliable detection of carriers of the mutated ITGA10 gene in canine population (Ensembl: ENSCAFG00000011346.4). On the basis of *in silico* analysis, the authors of this paper propose the following diagnostic scheme, which enables detection of heterozygous specimens. The first step is amplification of the COL11A2 gene sequence (Ensembl: ENSCAFG00000000903.4) with DNA-fragment flanking primers from 3361. to 3569. nt of the sequence – forward: 5'CCTGACTCTCATGGACTTTC3', reverse: 5'CCTCCTGGTGTCTGATTCTA3'. The resulting product lenght is 249 pz. The analysis of restriction fragment lenght polymorphism (PCR-RFLP) can be carried out with the BsoBI restriction enzyme from *Bacillus stearothermophilus* JN2091 bacteria; this enzyme recognizes 5'C|YCGRG3' sequence. NebCutter program was used to find restriction sites typical of the mutated sequence (Fig. 6); the wild sequence does not show any cutting sites which are recognized by this enzyme.

Using of the proposed restriction enzyme allows for separation of dominant and recessive homozygotes, as well as heterozygous specimens after their electrophoretic separation, as presented on Figure 7.

Miniature Poodle

Also in this breed causes of chondrodysplasia remain unknown, though some information suggest mutation as the cause of osteochondrodysplasia in this breed. This disease belongs to a broad group of bone and cartilage disorders, resulting from structural, metabolic and hormonal abnormalities. It was described some 60 years ago, yet its genetic background was explained only in 2012 [Neff et al. 2012]. It is caused by deletion of large DNA fragment comprising 14. and 15. exons of SLC13A1 gene. This gene encodes sodium-dependent sulphate transporter protein and remains the principal regulator of serum sulphate levels; sulphation of proteoglycans in extracellular matrix of cartilage is essential to skeletal development [Neff et al. 2012].

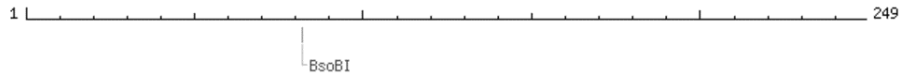

Fig. 6. Restriction site characteristic for BsoRI in the sequence of the amplified ENSCAFG00000000903.4 gene

Rys. 6. Miejsce restrykcyjne charakterystyczne dla enzymu BsoRI w sekwencji namnożonego fragmentu genu ENSCAFG00000000903.4

	Homozygote, 2x wild allele Homozygota, 2x allel dziki	Heterozygote Heterozygota	Homozygote, 2x mutated allele Homozygota, 2x allel zmutowany
249 bp (pz)	▬	▬	
166 bp (pz)		▬	▬
83 bp (pz)		▬	▬

Fig. 7. Electrophoretic patterns of different genotypes (dominant homozygote, recessive homozygote, heterozygote) as obtained by the authors after in silico analysis

Rys. 7. Wzór rozdziału elektroforetycznego homozygoty dominującej, recesywnej oraz heterozygoty po analizie in silico przeprowadzonej przez autorów

Summing up, chondrodysplasia is a genetic disease that causes discomfort, pain and suffering in affected specimens. Studies on its genetic background will hopefully lead to developing reliable screening tests, enabling to eliminate both affected (homozygous) animals and heterozygous carriers from breeding. Moreover, data presented in this article show that in some cases the domestic dog cannot be considered an appropriate model organism in studies aimed at understanding the genetic background of similar congenital defects in humans.

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GENETYCZNE UWARUNKOWANIA CHONDRODYSPLAZJI U PSA DOMOWEGO (*CANIS LUPUS FAMILIARIS*) – ANALIZA *IN SILICO*

Streszczenie. Chondrodysplazja jest to zaburzenie o podłożu genetycznym związane z nieprawidłowym rozwojem tkanki chrzęstnej. Chore osobniki charakteryzują się karłowatością kończyn oraz rozrostem kości czaszki, jednakże pierwsze objawy choroby pojawiają się dopiero w czasie wzrostu zwierzęcia. Dotychczas prowadzone badania wykazały autosomalny, recesywny charakter chondrodysplazji u psa domowego, dlatego też jedynym sposobem umożliwiającym wykrycie nosicieli w populacji bez prowadzenia kojarzeń testowych są testy genetyczne. Obecnie znane jest podłoże genetyczne chondrodysplazji tylko u psów rasy elkhund szary i karelski pies na niedźwiedzie, a w przypadku innych ras, np. labrador retriever dotychczasowe badania jedynie wykluczyły udział niektórych genów w warunkowaniu tej choroby. Dlatego autorki artykułu zdecydowały się na zaproponowanie genetycznych testów diagnostycznych umożliwiajacych weryfikację nosicieli na podstawie stwierdzonych dotychczas mutacji powodujących chondrodysplazje.

Słowa kluczowe: chyndroplazja, pies domowy, labrador retriever, COL11A2, elkhund szary, ITGA10

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