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Original Article

The association of genetic polymorphisms of bone formation genes with canine hip dysplasia

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Abstract

Background: Canine hip dysplasia (CHD) is an orthopedic disorder characterized by abnormal laxity of the hip joint. It is considered multifactorial and polygenic and affects predominantly medium and large sized dog breeds. **Aims:** The aim of this study was to identify CHD associated polymorphisms in chromosomal regions on CFA19, CFA24, CFA26, and CFA34. **Methods:** Blood samples from 60 dogs of different breeds were collected and genotyped, including 46 cases and 14 controls. After sequencing and single nucleotide polymorphism (SNP) determination of the target regions, an individual SNP analysis with a χ^2 statistic was performed based on the comparison of allele frequencies in cases and controls. **Results:** A significant association was observed between CHD and a T/C SNP on CFA19, which harbors genes involved in bone metabolism. No other significant association was found in the study and previously identified SNPs cannot be validated as related to CHD. **Conclusion:** Further research is warranted to identify CHD-associated polymorphisms in order to develop a genotype-based diagnosis and selection approach.

Key words: Association, Dog, Hip dysplasia, Selection, SNP

Introduction

Canine hip dysplasia (CHD) is a common genetic disorder, which mainly affects medium and large sized-breeds. Canine hip dysplasia occurs because of an abnormal formation of the hip joint due to a defect in bone tissue development (Kaneene *et al*., 2009). Former researches have indicated that CHD has a polygenic inheritance pattern and also many environmental factors influence the pathogenesis of the disease. This orthopedic condition characterized by the malformation of coxo-femoral joint causes instability and subluxation of the hip, which can lead to osteoarthritis and lameness. Breed prevalence ranged from 1 to 75% indicates the strong genetic component (Janutta *et al*., 2006). As one of the most common developmental skeletal disorders, CHD cannot be cured, although it can be improved by surgery and dietary regulations. Due to the difficulty of the treatment, genetic approaches should be explored based on the hereditary character of CHD (Fries and Remedios, 1995; Lewis *et al*., 2010). According to the results of several studies, selective breeding programs based on genetic information, rather than phenotypic information, may have more success in getting improvement (Stock and Distl, 2010; Guo *et al*., 2011; Sánchez-Molano *et al*., 2013).

Until recently, breeding programs against CHD were mainly based on the phenotypic information, which is focused on radiographic screening of the pelvic area. A hip score is provided by the evaluation of the radiographs based on nine different traits (Willis, 1997; Lewis *et al*., 2010). Three of these traits are highly heritable and are related to joint laxity (Norberg Angle), subluxation and cranial acetabular edge. Nevertheless, this approach provided limited success (Willis, 1997; Hou *et al*., 2013). Therefore, studies conducted in recent years focused on discovering significant single nucleotide polymorphisms (SNPs), quantitative trait loci (QTL) and candidate genes for CHD. The most association studies were conducted on Labrador Retrievers (Sánchez-Molano *et al*., 2015), German Shepherd dogs (Maki *et al*., 2004; Janutta *et al*., 2006; Marschall and Distl, 2007; Fels and Distl, 2014), Portuguese Water dogs (Chase *et al*., 2004; Chase *et al*., 2005), Labrador Retriever-Greyhound crossbred dogs (Todhunter *et al*., 2005), Bernese Mountain dogs (Pfahler and Distl, 2012), Golden Retrievers (Lavrijsen *et al*., 2014), Newfoundland dogs and Rottweilers (Zhou *et al*., 2010).

Previous researchers reported a number of SNPs associated with the increased risk for CHD. One of the SNPs found to be associated with CHD risk was identified in *fibrillin-2* gene (FBN2), this gene encodes an extracellular matrix component present in the joint capsule and articular cartilage (Friedenberg *et al*., 2011).

In a genome-wide association study (GWAS) on functional genes playing a role in extracellular matrix development, a number of SNPs associated to CHD were identified inside those genes or close to them (Lavrijsen *et al*., 2014). In a study aiming to develop a genetic predictive model based on seven SNPs, researchers found two strongly CHD associated SNPs. One of them is 14 bp upstream and the other one is 1051 bp upstream carbohydrate chondroitin 6 sulfotransferase gene (CHST3), which affects chondroitin sulfate biosynthesis (Bartolome *et al*., 2015). A QTL identification study conducted on German Shepherd dogs validated five CHD-associated SNPs in CFA19, CFA24, CFA26, and CFA34. Three SNPs in CFA24, CFA26, and CFA34 are inside or close to genes, which are involved in bone formation, osteoclast activity, chondrocyte proliferation and differentiation, namely *SRC* gene [v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog], kinase suppressor of ras 2 (KSR2) and triple functional domain (TRIO) genes (Fels and Distl, 2014).

The aim of the present study was to identify the genetic variations in four chromosomal regions, CFA19, 24, 26, and 34, which harbor or are in close proximity to genes involved in bone formation and determine their associations with CHD.

Materials and Methods

Animals

The study was carried out using 46 CHD-affected and 14 unaffected dogs. All animals presented in Istanbul University, Faculty of Veterinary Medicine, Department of Surgery between years 2014 and 2016. The animals were unrelated. The characteristics and CHD scores of the dogs were given in Table 1. After a physical examination, dogs were X-rayed and CHD scores were determined based on the Norberg Angle measured according to the ventro-dorsal hip radiographs. Canine hip dysplasia scores, sex, age, and breed information were recorded. For DNA extraction 5 ml of blood samples were collected into vacuumed tubes containing EDTA. The study was approved by the "Istanbul University Animal Researches and Ethics Committee" with the verdict number 2014/68.

DNA extraction, polymerase chain reaction (PCR) and sequencing

Genomic DNA samples were isolated from whole blood by using the standard salt-out method (Miller *et al*., 1988). Target regions in CFA19, CFA24, CFA26, and CAF34 were amplified. Chromosomes, primers, annealing temperatures, and product sizes were given in Table 2. Polymerase chain reaction amplifications were performed in a reaction volume of 25 µL using 1 U *Taq* polymerase, 2-2.5 µL 10XPCR buffer [100 mM KCl, 20 mM Tris HCl (pH = 8.0), 0.1 mM Ethylenediaminetetraacetic acid (EDTA), 0.5 mM Phenylmethylsulfonyl fluoride (PMSF), 1 mM DTT, 50% glycerol], 2.5 mM $MgCl_2$, 50-100 ng genomic DNA, 100 µ MdNTP and 10 pmol of each primer. Amplifications were carried out with an initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 60 s, primer-specific annealing temperature for 60 s, 72°C for 60 s; and a final extension at 72°C for 10 min. The PCR products were run through 2% agarose gel to check the results of amplification. After the purification of the PCR products, sequencing was performed by using an ABI-3100 sequencer (PE Biosystems, Germany) and the BigDyeTM terminator cycle sequencing kit (ThermoFisher Scientific, USA).

Determination of sequence variations

Nucleotide sequences of all amplified regions were aligned by using ClustalW program in the MEGA 6 software (Tamura *et al*., 2013). Single nucleotide polymorphisms and other variations were determined. The previous reported SNPs were obtained from CanFam 3.0 for comparison.

Statistical analysis

Allele and genotype frequencies of SNPs were calculated for dogs with CHD and unaffected animals by using SPSS 20.0 program. The comparison of allele and genotype frequencies in cases and unaffected dogs was made to examine whether a polymorphism is significantly associated with CHD. An association test for SNP frequencies with CHD was performed by using Pearson's two-sided Chi-square (χ^2) test in SPSS 20.0 program. Odds ratios (OR) with 95% confidence intervals (CI) were estimated by using unconditional logistic regression.

Results

Partial sequencing of CFA19, CFA24, CFA26, and CFA34 revealed 13 polymorphisms containing 12 SNPs and one 10 bp insertion/deletion polymorphism. The genetic polymorphisms determined in this study are available in the European Variation Archive under project accession ID PRJEB24783. Nucleotide changes, chromosomal positions and minor allele frequencies (MAF) and values indicating allele associations were presented in Table 3. Minor allele frequencies of these polymorphisms and OR varied between 0.16-0.48 and 0.134-2.88, respectively.

Among all the polymorphisms, the association between SNP T/C in CFA19 and CHD was found to be statistically significant ($P \leq 0.05$). The genotypes were shown in Figs. 1A-C. Other five SNPs found in CFA19 had the same allele frequencies, indicating that they tend to be inherited together as a haplotype. Four SNPs and one in/del polymorphism (ACT GGA CAC T) were identified in CFA24. Partial sequencing of CFA26 and CFA34 revealed only three previously identified SNPs, namely one C/T transition and C/T, A/G transitions in CFA26 and CFA34, respectively. No novel polymorphism was observed. None of the SNPs are located in coding regions. Except for the SNP in CFA26 located in intron 5 of the *KSR2* gene, other polymorphisms are intergenic.

Table 1: Information and CDH score of the dogs in case and control groups

Case No.	Breed	Age	Sex	CHD score
1	Samoed	12 months	Male	E
2	Labrador	6 months	Male	B
3	Labrador	5 years	Female	C
4	Kangal	16 months	Female	E
5	German Shepherd dog	6 months	Male	D
6	Rottweiler	11 months	Female	E
7	German Shepherd dog	9 months	Female	E
8	Labrador Retriever	9 months	Female	D
9	Labrador Retriever	9 months	Male	B
10	Mixed	12 months	Male	D
11	Kangal	12 months	Male	D
12	German Shepherd dog	9 months	Male	C
13	Setter	3 years	Male	C
14	German Shepherd dog	2 years	Male	D
15	Bernese Mountain dog	13 months	Male	D
16	Rottweiler	12 months	Female	D
17	Dogo Argentino	5 years	Male	D
18	Setter	18 months	Female	E
19	Mixed	2 years	Female	E
20	Golden Retriever	8 years	Male	D
21	Rottweiler	7 months	Male	D
22	Kangal	9 months	Male	D
23	German Shepherd dog	5 years	Female	E
24	Rottweiler	6 months	Male	E
25	German Shepherd dog	18 months	Male	D
26	Golden Retriever	12 years	Male	D
27	Kangal	12 months	Male	C
28	Labrador Retriever	10 years	Female	E
29	Pug	3 years	Male	C
30	English bulldog	12 months	Male	B
31	Setter	4 years	Male	B
32	Labrador Retriever	12 years	Female	E
33	Golden Retriever	12 months	Male	D
34	Labrador Retriever	12 months	Male	D
35	Setter	9 years	Male	E
36	Kangal	10 months	Female	D
37	Chow-chow	18 months	Female	E
38	Golden Retriever	6 months	Male	E
39	German Shepherd dog	5 years	Male	C
40	Kangal	2 years	Female	E
41	Rottweiler	8 months	Male	C
42	Golden Retriever	10 months	Male	D
43	Golden Retriever	10 years	Female	E
44	Belgian Shepherd dog	5 months	Male	D
45	Labrador Retriever	6 years	Male	E
46	Rottweiler	8 years	Female	E
C1	Rottweiler	3 months	Male	A
C2	Golden Retriever	7 months	Female	A
C3	Kangal	5 months	Male	A
C4	Mixed	4 years	Male	A
C5	Labrador Retriever	18 months	Female	A
C6	Mixed	18 months	Female	A
C7	Golden Retriever	7 years	Female	A
C8	Pitbull	13 years	Female	A
C9	Husky	3 years	Female	A
C10	German Shepherd dog	13 years	Female	A
C11	Golden Retriever	14 years	Male	A
C12	Golden Retriever	8 years	Female	A
C13	Labrador Retriever	10 years	Male	A
C14	German Shepherd dog	7 months	Male	A

CHD: Canine hip dysplasia, C1-C14: Samples of control group, A: Normal hip joint, B: Near normal hip joints, C: Mild hip dysplasia, D: Moderate hip dysplasia, and E: Severe hip dysplasia

Table 2: Primer sequences, annealing temperatures and product size of the amplified regions

CFA	Primer sequence (5´-3´)	Annealing temperature	Product size (bp)
19	F: CTGCACCATAGTCCAGACA	61	469
	R: ATGTTTACAAGTGTGGTCCAAG		
24	F: GAGAGGACTGAAATAAAGCATTTAGC	62	471
	R: CTCAGAACGCTTCTCGACAC		
26	F: ACTCCCAGACCAGGAACA	62	468
	R: ATTCCAAGGCTAACCCAGTG		
34	F: TGCTAGGAACTCTCTCTCTTC	60	447
	R: CCCTTCTGAGACTACACAGTA		
34	F: TAGCCACGTTTGAGATCATGG	62	432
	R: GAGGTTTGTGTCCATATCCGT		

CFA: Chromosome of *Canis familiaris*

Table 3: Association of the polymorphisms to CHD

Polymorphism	CFA	Alleles	Genome position[a]	Minor allele	MAF		OR (95% CI)	χ^{2b}
					Total	CHD		
This study[c]	19	T/C	32513672	C	0.48	0.53	2.88 (0.94-8.86)	3.60*
This study	19	A/G	32513714	G	0.48	0.47	0.66 (0.24-1.85)	0.39
This study	19	A/C	32513715	C	0.48	0.47	0.66 (0.24-1.85)	0.39
TIGRP2P265674	19	A/G	32513724	G	0.48	0.47	0.66 (0.24-1.85)	0.39
This study	19	G/A	32513796	A	0.48	0.47	0.66 (0.24-1.85)	0.39
This study	19	G/A	32513801	A	0.48	0.47	0.66 (0.24-1.85)	0.39
This study	24	G/A	25973355	A	0.16	0.17	1.67 (0.34-8.17)	0.39
BICF2S2367279	24	G/C	25973438	C	0.44	0.33	0.55 (0.19-1.54)	1.33
This study	24	C/T	25973518	T	0.40	0.36	0.44 (0.16-1.25)	2.45
This study	24	in/del	25973563-25973572	in	0.35	0.32	0.60 (0.14-2.65)	0.46
BICF2P281364	26	C/T	14157064	T	0.23	0.23	0.90 (0.22-3.68)	0.02
BICF2P1086886	34	C/T	1223586	T	0.44	0.45	1.36 (0.45-4.10)	0.30
BICF2P355865	34	A/G	36340417	G	0.32	0.33	1.30 (0.42-4.01)	0.21

[a] Position on dog chromosome obtained from genome assembly, CanFam3.1 (GCA_000002285.2), [b] Pearson's two sided Chi-square test, and[c] The genetic polymorphisms determined in this study are available in the European Variation Archive under project accession ID PRJEB24783. * P≤0.05. CFA: Chromosome of *Canis familiaris*, MAF: Minor allele frequencies, CHD: Canine hip dysplasia, OR: Odds ratio, and CI: Confidence intervals

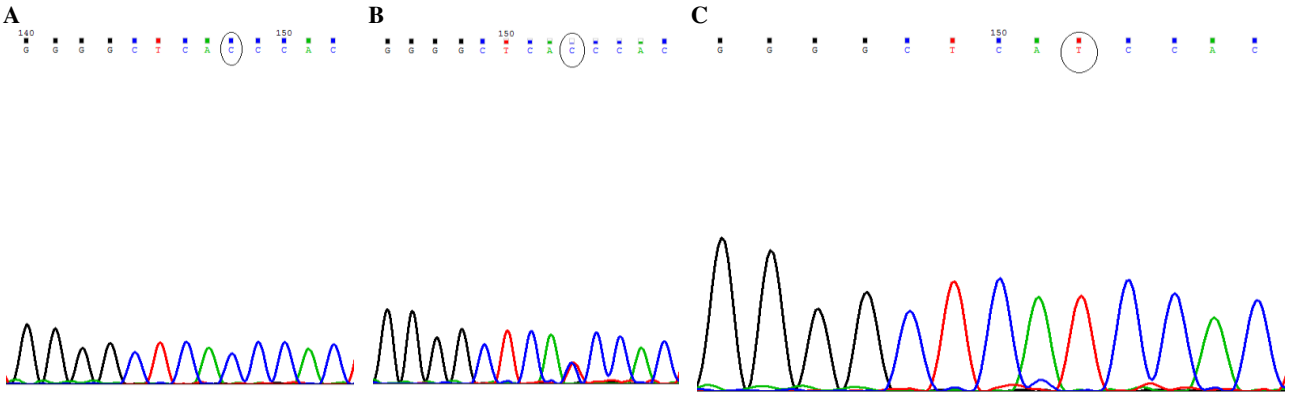

Fig. 1: The genotypes of the C/T SNP on CFA19 on genome position 32.513.672 (**A**) CC homozygote, (**B**) CT heterozygote, and (**C**) TT homozygote

Discussion

Canine hip dysplasia is a common orthopedic disorder with a high prevalence in medium and large-sized breeds, which highlights the genetic basis of this disorder (Lewis *et al*., 2010). In recent years several studies were performed on the prediction of CHD by using genetic markers in order to prevent the disorder by genotype-based selective breeding of dogs (Fels and Distl, 2014; Lavrijsen *et al*., 2014; Sánchez-Molano *et al*., 2014; Bartolome *et al*., 2015). In the present study, we focused on four chromosomal regions which are found to be associated with CHD in German Shepherd dogs. Different breeds of medium and large sized dogs were included in the study. In statistical analysis, the grade of CHD was not taken into account due to the limited number of samples.

Among the SNPs and one in/del polymorphism identified in this study, only T/C transition in position 32.513.672 on CFA19 was found to be associated with CHD (P<0.05). In a GWAS on 1035 German Shepherd dogs conducted by Fels and Distl (2014), CHD-

associated SNPs except for this T/C polymorphism in the position 32.513.672 were determined on CFA19. Existence of breed-specific polymorphisms may have revealed these results. There are two genes located on CFA19, which are involved in bone metabolism; *FGF2* (fibroblast growth factor 2) gene and *NUDT6* (nudix hydrolase) gene. *FGF2* gene has effects on osteoblast differentiation and bone formation (Nakano *et al*., 2015). *NUDT6* gene has a regulatory role on FGF2 expression (Baguma-Nibasheka *et al*., 2012). T/C polymorphism identified in this study may have a link to these genes. In further studies FGF2 in particular can be considered as a candidate gene for CHD.

In the same study, Fels and Distl (2014) identified one SNP on each of the chromosomes CFA24 and CFA26 and two SNPs on CFA34 associated with CHD. Same SNPs were also observed in our study but we found no evidence for the association for these polymorphisms. In addition to the difference of genetic background of the breeds, the limited number of samples may also have revealed these results.

The region on CFA24 analyzed in this study presented a high polymorphism with four SNPs and one in/del polymorphism. This region is in close proximity to *SRC* (v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog) gene. This gene has a role in bone formation. Due to its effects on cytoskeletal reorganization and osteoclast activity, disruption of SRC in mice caused osteopetrosis (Boyce *et al*., 2006; Miyazaki *et al*., 2006).

In a study on UK Labrador Retrievers four genome-wide significant SNPs and 73 chromosome-wide significant SNPs were observed. One of the chromosome-wide significant SNPs is located on CFA24 (Sánchez-Molano *et al*., 2014). CFA24 seems to be important due to its high polymorphism and SNP content associated with CHD in different dog breeds. Further studies with a higher number of samples on a variety of breeds may reveal new links to CHD.

Kinase suppressor of ras 2 and *TRIO* genes are located on CFA26 and CFA34, respectively. Kinase suppressor of ras 2 is involved in osteoblastogenesis (Liu *et al*., 2009), and TRIO has an important function in chondrogenic proliferation and differentiation (Wang and Beier, 2005). A study on German Shepherd dogs validated *KSR2* and *TRIO* genes as candidate genes for CHD (Fels and Distl, 2014). We did not observe any association between the SNPs identified in CFA26, CFA34, and CHD. The validity of these two genes as candidate genes should be explored in other dog breeds.

Looking at the results of the association analyses, it is seen that the differences are not only between breeds, but also between populations belonging to the same breed. A deletion in an intron of *FBN2* causing suppression of the gene, was found to be associated with CHD in Labrador Retrievers from the USA (Friedenberg *et al*., 2011). But no association was observed between this gene and CHD in a Labrador Retriever population from Holland. The researchers reported that two populations from the USA and Holland have diverged according to population stratification analysis (Lavrijsen *et al*., 2014).

Up-to-date GWASs were mainly conducted on Labrador Retriever and German Shepherd dog breeds. Canine hip dysplasia is a disorder with a significant breed predisposition but is also influenced by metabolic and environmental factors (Janutta *et al*., 2006). Genetic differences between breeds and populations may also contribute to the complex etiology of CHD, thus enhancing the importance of validity check of CHD associated SNPs in different dog breeds and populations. For selective breeding, a SNP panel valid for different breeds would be more efficient. A predictive genetic test for early diagnosis of hip dysplasia based on seven SNPs was developed in Labrador Retrievers from Spain. Most of the SNPs were found to be located near genes involved in bone metabolism. Authors of the study reported that further research is needed to evaluate the validity of the test in other dog breeds (Bartolome *et al*., 2015).

In recent studies many candidate genes on different chromosomal regions were proposed to be associated with CHD. Findings from these studies suggest a genetic basis composed of many genes with low or moderate effects based on their roles in different metabolic processes (Lavrijsen *et al*., 2014; Sánchez-Molano *et al*., 2014; Bartolome *et al*., 2015). Further analysis of these genes would contribute to the database, which can be used for genetic elimination of the disorder. Due to the complex nature of CHD inheritance, genomic selection approach may be more effective compared to marker-assisted selection (Sánchez-Molano *et al*., 2013).

In conclusion, one SNP on CFA19 located near genes involved in bone metabolism was found to be associated with CHD. On the other hand, associations of SNPs, which are proposed to be related to the CHD in the past studies, cannot be validated. Regarding breeding strategies based on genotype-based selection, further research is warranted to identify a sufficient number of polymorphisms with adequate coverage in different dog breeds.

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Conflict of interest

The authors declare that there is no conflict of interest.

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