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RESEARCH ARTICLE

Detection of DNA Markers in Dogs with Patellar Luxation by High Annealing Temperature - Random Amplified Polymorphic DNA Analysis [1]

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Summary

Patellar luxation is one of the orthopaedic disorders found mostly in small-breed dogs. It can be inherited by the next generation, causing continuing problems in the dogs' health. However, if it can be detected, the affected dogs will not be selected for breeding, and hence the incidence will be less. In this study, the objective was to find a DNA marker representing dogs with patellar luxation which can be detected when first born. High annealing temperature-random amplified polymorphic DNA (HAT-RAPD) technique was used to amplify 39 dog blood samples (16 unaffected and 23 affected). It was also used to develop the polymorphic fragments capable of distinguishing patellar-luxation-affected dogs from those unaffected. Three candidate fragments were sequenced and found to be parts from three different chromosomes (10, 36 and X) after comparison with the GenBank dog genome database using the BLAST algorithm. Association analysis was performed using a chi-square test. The results showed that the fragment (generated by the OPB05 primer) from chromosome 36 was potentially related to the two groups of dogs, with a P value of 0.042. This is the first finding of a gene which related to canine patellar luxation, and merits further investigation.

Keywords: *Patella luxation, Dog, HAT-RAPD, DNA marker*

Patella Çıkığı Olan Köpeklerde DNA Belirteçlerinin Yüksek Bağlanma Sıcaklıklı RAPD Analizi ile Belirlenmesi

Özet

Patellar luksasyon küçük ırk köpeklerde en yaygın gözlenen bozukluklardan birisidir. Sonraki nesillere aktarılmak suretiyle köpeklerde süregelen bir problem olabilir. Ancak tespit edilmesi durumunda etkilenmiş köpekler üretim amaçlı kullanılmaz ve böylece görülme sıklığı azaltılabilir. Bu çalışmada, patellar luksasyon ile doğan köpeklerin tespitinde kullanılmak amacıyla bir DNA markır bulmak amaçlanmıştır. High annealing temperature–random amplified polymorphic DNA (HAT-RAPD) tekniği ile 39 köpeğin (16 normal, 23 patellar luksasyonlu) kan örnekleri kullanıldı. Üç aday parçacık (fragment) sekanse edildi ve BLAST algoritması kullanılarak Den Bank köpek genomu ile karşılaştırıldığında bunların üç farklı kromozomdan (10, 36 ve X) bölümler olduğu tespit edildi. Asosiasyon analizi chi-kare testi kullanılarak yapıldı. Sonuçlar 36. kromozomdaki parçacığın (OPB05 primeri ile üretilen) potansiyel olarak iki grup köpek ile ilgili olduğunu ortaya koydu (P=0.042). Bu çalışma köpek patellar luksasyon ile ilgili bir gene ilişkin ilk çalışma olup gelecek araştırmalara ihtiyaç vardır.

Anahtar sözcükler: *Patella Luksasyon, Köpek, HAT-RAPD, DNA belirteci*

INTRODUCTION

Various diseases and ailments are frequently found in dogs; some are fatal, while others can cause pain and affect their daily lives. Some breeds of dogs are prone to certain genetic ailments, such as elbow or hip dysplasia,

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blindness, and patellar luxation [1]. Especially in recent years, patellar luxation problems appear to be getting worse in Pomeranian and other small breeds. It has also been reported that this can be inherited polygenically [2,3], making it a serious problem that small dog breeders should address so that future generations of dogs will have strong patellas and hind limbs. Based on a recent health survey from 1974-2011, 40% of Pomeranians were diagnosed with patellar luxation [4]. Small dogs (weighing less than 9 kg) are said to be 12 times more likely to be affected by patellar luxation than medium, large or giant dogs. It can also be a heartbreaking problem for puppy purchasers. As a consequence, it is highly recommended that dogs with this disorder should not be used for breeding purposes [5]. However, because affected dogs rarely show symptoms until they enter middle age, at an early stage of patellar luxation it might be difficult to prognosticate whether some dogs will develop the disorder [6]. In cases of congenital patellar luxation, dogs carrying this trait can theoretically be detected by genetic markers, leading to a reduction in its incidence. Thus, the objective of this study was to construct a sequence-characterized amplified region (SCAR) marker derived from DNA polymorphisms in the genome of dogs with patellar luxation for discrimination and prediction of this disorder.

MATERIAL and METHODS

Blood Samples

Thirty-nine canine blood samples *(Table 1)* were kindly provided by a small animal clinic in Chiang Mai province. All samples were collected from a variety of small breeds and ages and were kept at −20°C, without any anticoagulant, prior to the experiment.

DNA Extraction

The isolation of total genomic DNA for molecular marker analysis was carried out utilizing the phenol-chloroform method of Taş [7].

HAT-RAPD Analysis

Genomic DNA from each sample was diluted to a concentration of 10 ng/ml. The amplifications followed the protocol of Anuntalabhochai et al.[8] and Liu et al.[9]. PCR was performed in a total volume of 25 µl containing: 1X reaction buffer (500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl, 1 mg/ml BSA, and 100 mM $(NH_4)_2SO_4$; RBC Bioscience, Taipei, Taiwan); 2 mM $MgCl_2$ (RBC Bioscience); 0.2 mM dNTP (Vivantis Technologies, Malaysia); 0.4 µM primers *(Table 2)* (Operon Technologies, Alameda CA, USA); 1 U *Taq* DNA polymerase (RBC Bioscience); 10 ng/ml genomic DNA; and deionized distilled water. PCR was performed using an MJ Mini Personal Thermal Cycler (Bio-Rad Laboratories, Hercules CA, USA) with the following cycling profile: 1 cycle at 95°C for 5 min; 35 cycles at 94°C for 30 sec, 45°C for 1 min and 72°C for 1.5 min; and a final cycle at 72°C for 5 min. After PCR was completed, the amplified samples were kept at 4°C prior to agarose gel electrophoresis.

Cloning of DNA Fragments

Candidate fragments were excised, using a clean scalpel, and purified using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany) following the manufacturer's recommended protocol for DNA extraction from agarose gel. The candidate fragments were ligated with the RBC TA Cloning Vector Kit using T_4 DNA ligase enzyme (RBC Bioscience), following the manufacturer's recommended protocol. The fragment-ligated vectors were transformed into HIT-DH5α competent cells (RBC Bioscience) following the manufacturer's recommended protocol. The mixture was incubated on ice for 10 min and poured immediately onto previously prepared luria broth (LB) agar. Positive colonies were selected by blue/white colony selection technique and were confirmed for the inserted DNA fragments by colony PCR using M13 forward and reverse primers. A colony that gave the correct DNA fragment length was inoculated in LB broth with vigorous shaking (200 rpm) at 37°C overnight or until the OD_{600} reached 0.5-0.7. The plasmid was extracted using the PureYield™ Plasmid Miniprep System (Promega, Fitchburg WI, USA) following the manufacturer's recommended centrifugation protocol.

Sequencing and Sequence Identification

The sequences of all fragments were commercially analyzed by an automated sequencer (1st BASE, Singapore) and compared with the GenBank dog *(Canis lupus familiaris)* genome database using the BLAST algorithm [10]. The likelihood was considered to correspond to the E-value, which had to be less than 1^{-30} to indicate a significant similarity.

Association Analysis

The relationships between the polymorphic fragments and the two populations were analyzed using a chi-square test. The appearance of each polymorphic fragment was scored as 0 or 1 for its occurrence or absence, respectively. The data were used to calculate the χ^2 value, a *P* value of <0.05 was considering significantly difference.

RESULTS

Polymerase chain reaction (PCR)-based high annealing temperature-random amplified polymorphic DNA (HAT-RAPD) was performed using 16 primers *(Table 2)* on pooled DNA of 39 canine blood samples to select high polymorphic primers. The results are shown in *Fig. 1* and *Table 2*. These primers yielded a total number of 91 amplified fragments and 294 polymorphic fragments. The fragments ranged in size from 300 to 3.500 base pairs. In a search

Table 1. *Data from 39 canine blood samples (Numbers in parentheses represent patellar luxation grading; M = male, F = female)*
Table 1. *39 adet köpek kan örneğine ait veriler (parantez içerisindeki numaralar patellar luksasyon derecesini göstermektedir;* M = erkek, F = dişi)

Sample Code	Breed	Sex	Age (months)	Weight (kg)	Patellar Luxation
PL01	Chihuahua	F	6	3.6	Affected (3/4)
PL02	Chihuahua	F	7	2.5	Affected (2/4)
PL03	Chihuahua	M	7	3.2	Affected (3/4)
PL04	Chihuahua	F	8	1.2	Affected (2/4)
PL05	Chihuahua	F	9	2.25	Affected (2/4)
PL06	Chihuahua	M	9	3	Affected (3/4)
PL07	Chihuahua	M	13	2.3	Affected (4/4)
PL08	Chihuahua	F	14	2	Affected (3/4)
PL09	Pekingese	M	5	5.4	Affected (3/4)
PL10	Pomeranian	M	6	1.2	Affected (3/4)
PL11	Pomeranian	M	7	4	Affected (3/4)
PL12	Pomeranian	F	8	1.4	Affected (4/4)
PL13	Pomeranian	M	8	5	Affected (4/4)
PL14	Pomeranian	F	11	3	Affected (4/4)
PL15	Pomeranian	M	14	3.5	Affected (3/4)
PL16	Pomeranian	F	14	14	Affected (3/4)
PL17	Poodle	F	8	3.2	Affected (3/4)
PL18	Poodle	F	8	3.5	Affected (2/4)
PL19	Poodle	F	10	3.4	Affected (3/4)
PL20	Poodle	F	12	1.9	Affected (2/4)
PL21	Poodle	F	13	1.2	Affected (4/4)
PL22	Poodle	F	18	2.1	Affected (2/4)
PL23	Yorkshire Terrier	F	7	1.2	Affected (3/4)
N01	Chihuahua	F	19	1.4	Unaffected
N02	Chihuahua	F	25	2.4	Unaffected
N03	Chihuahua	F	32	2.4	Unaffected
N04	Chihuahua	M	54	3.9	Unaffected
N05	Chihuahua	M	60	1.8	Unaffected
N06	Chihuahua	F	70	2.4	Unaffected
N07	Chihuahua	F	98	3	Unaffected
N08	Pomeranian	F	54	9.2	Unaffected
N09	Pomeranian	F	75	2.8	Unaffected
N10	Pomeranian	F	83	3	Unaffected
N11	Pomeranian	M	98	3	Unaffected
N12	Poodle	M	13	7	Unaffected
N13	Poodle	F	23	2.85	Unaffected
N14	Shih Tzu	M	42	3.2	Unaffected
N15	Shih Tzu	F	84	5.8	Unaffected
N16	Yorkshire Terrier	F	32	1.85	Unaffected

of polymorphic fragments in two sample groups (non-affected and affected by patellar luxation), PCR-based HAT-RAPD was done with 16 randomly chosen primers of arbitrary nucleotide sequences on two DNA templates, pooled from both groups of samples. The results are shown in *Fig. 2* with arrows indicating two polymorphic fragments found. Two primers (OPB05 and R105) that gave the polymorphic fragments were chosen to amplify all 39 DNA samples. The results are shown in *Fig. 3* and *4*, respectively. The P values of the relationship between the two populations and the OPB05 and R105 primer-generated polymorphic fragments were 0.042 and 0.112,

Table 2. *List of primers and their sequences used in HAT-RAPD analysis, number of fragments and size ranges*

Table 2.*HAT-RAPD analizinde kullanılan primerlerin ve sekanslarının, parçacık numaraların ve boyutlarının listesi*

Primer	Sequence (5′ 3′)	Total Number of Amplified Fragments	Fragment Size Range (bp)
1. A4	GCATCAATCT	7	300-3.000
2. AP42	AACGCGCAAC	6	200-3.000
3. OPB04	GGACTGGAGT	4	500-2.000
4. OPB05	TGCGCCCTTC	5	600-3.000
5. OPB06	TGCTCTGCCC	3	800-2.000
6. OPB07	GGTGACGCAG	7	550-3.500
7. OPB08	GTCCACACGG	6	500-3.000
8. OPB10	CTGCTGGGAC	5	700-3.000
9. OPB17	AGGGAACGAG	9	300-4.000
10. OPB18	CCACAGCAGT	7	200-4.000
11. OPS11	AGTCGGGTGG	8	200-3.000
12. OPS16	AGGGGGTTCC	3	600-2.000
13. OPW09	GTGACCGAGT	5	450-2.500
14. R37	GAGTCACTCG	5	800-2.500
15. R55	CGCATTCCGC	5	220-3.800
16. R105	GCACCGAACG	6	550-2.200
Total		91	200-4.000

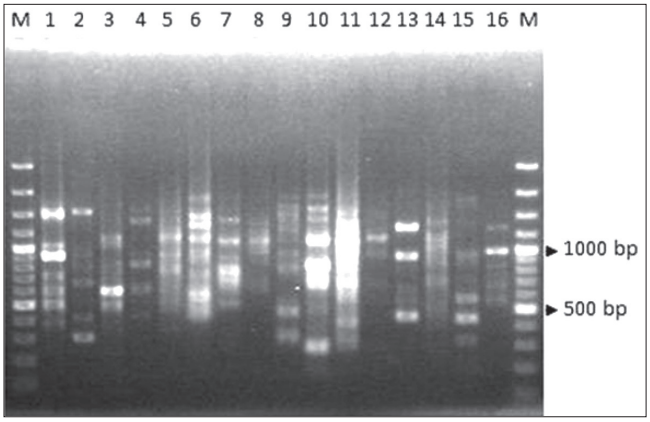

Fig 1. Two percent agarose gel stained with ethidium bromide and photographed by a UV transilluminator under UV light shows bands from random primer amplifications by 16 decanucleotide primers (1-A4, 2-AP42, 3-OPB04, 4-OPB05, 5-OPB06, 6-OPB07, 7-OPB08, 8-OPB10, 9-OPB17, 10-OPB18, 11-OPS11, 12-OPS16, 13-OPW09, 14-R37, 15-R55, and 16-R105)

Şekil 1. Etidiyum bromit ile boyanan %2'lik agar jel UV ışık altında fotoğraflanmıştır. 16 dekanükleotid primerler ile elde edilen rastgele primer amplikonlarını gösterir bantlar (1-A4, 2-AP42, 3-OPB04, 4-OPB05, 5-OPB06, 6-OPB07, 7-OPB08, 8-OPB10, 9-OPB17, 10-OPB18, 11-OPS11, 12-OPS16, 13-OPW09, 14-R37, 15-R55, ve 16-R105)

respectively, when calculated by a chi-square test (the test of independence) with a degree of freedom of 1. Based on a 95% confidence interval, the OPB05-generated fragment was significantly related to the disorder. The

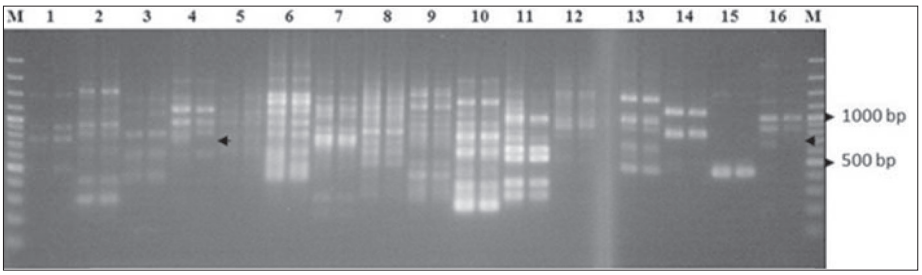

Fig 2. Two percent agarose gel stained with ethidium bromide and photographed by a UV transilluminator under UV light shows fragments from HAT-RAPD reactions with 16 randomly chosen primers (1-A4, 2-AP42, 3-OPB04, 4-OPB05, 5-OPB06, 6-OPB07, 7-OPB08, 8-OPB10, 9-OPB17, 10-OPB18, 11-OPS11, 12-OPS16, 13-OPW09, 14-R37, 15-R55, and 16-R105) of arbitrary nucleotide sequences on two DNA templates, pooled from samples non-affected and affected by patellar luxation. Arrows show polymorphic fragments that might be used to distinguish the two groups of samples

Şekil 2. Etidiyum bromit ile boyanan %2'lik agar jel UV ışık altında fotoğraflanmıştır. Patellar luksasyonlu ve normal hayvanların iki DNA eşi üzerindeki rastgele nukleotid sekanslarına ait 16 rastgele seçilmiş primer (1-A4, 2-AP42, 3-OPB04, 4-OPB05, 5-OPB06, 6-OPB07, 7-OPB08, 8-OPB10, 9-OPB17, 10-OPB18, 11-OPS11, 12-OPS16, 13-OPW09, 14-R37, 15-R55, ve 16-R105)kullanılarak HAT-RAPD reaksiyonu ile elde edilen fragmentler

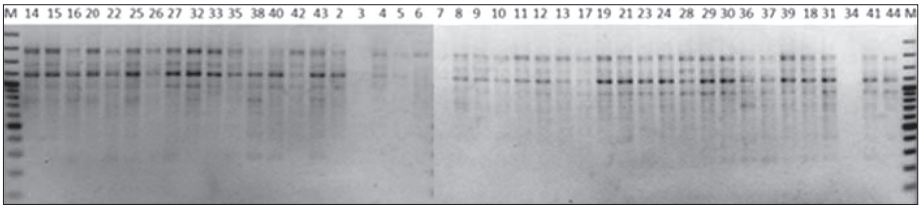

Fig 3. Two percent agarose gel stained with ethidium bromide and photographed by a UV transilluminator under UV light shows fragments amplified by HAT-RAPD reaction with OPB05 primer in DNA samples

Şekil 3. Etidiyum bromit ile boyanan %2'lik agar jel UV ışık altında fotoğraflanmıştır. DNA örneklerinde OPB05 primeri kullanılarak HAT-RAPD reaksiyonu ile amlife edilmiş fragmentler

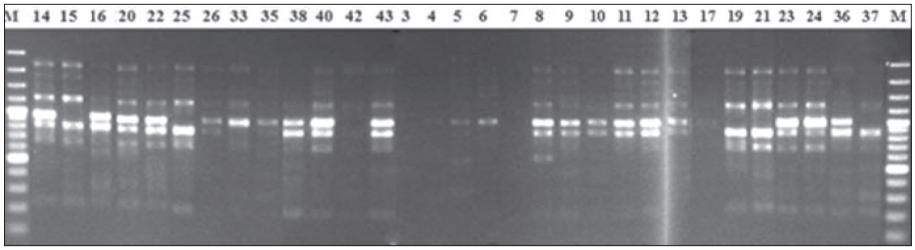

Fig 4. Two percent agarose gel stained with ethidium bromide and photographed by a UV transilluminator under UV light shows fragments amplified by HAT-RAPD reaction with R105 primer in DNA samples

Şekil 4. Etidiyum bromit ile boyanan %2'lik agar jel UV ışık altında fotoğraflanmıştır. DNA örneklerinde R105 primeri kullanılarak HAT-RAPD reaksiyonu ile amlife edilmiş fragmentler

sequences after alignment of three fragments from OPB05 (KF146953), R105-1 (KF146954) and R105-2 (KF146955) are shown in Additional file 1. Those three fragments were shown to be the genomic scaffolds from chromosomes 36, X and 10, respectively.

DISCUSSION

In this study, three different DNA fragments were found in the domestic dog genome, using HAT-RAPD analysis with 16 decanucleotide primers in an attempt to find molecular markers for canine patellar luxation. One fragment showed a significant relationship to the disorder, with a *P* value of 0.042. It has been suggested that canine patellar luxation is inherited [2], and patellar luxation has been demonstrated to be polygenic in its inheritance [3]. Although Soontornvipart et al.[11] showed 15 pedigrees of families of patellar luxation-affected dogs, no mode of inheritance has been confirmed as responsible for the penetrance of the phenotype. In addition, from the same study, microsatellite markers linked to the specifically collagen-related genes were used to identify the responsible polymorphism; however, the result indicated a non-involvement in the pathogenesis of patellar luxation, which supported the conclusion of Chase et al.[3] that patellar luxation is polygenic. Thus, the HAT-RAPD marker was chosen to identify the potential candidate polymorphisms accounting for the pathogenesis of patellar luxation, considering the ability of conventional RAPD to simultaneously screen several loci in the genome [12] and the high reproducibility of newly adapted HAT-RAPD [8].

Although HAT-RAPD has been used in plants [13,14] and platyhelminthes [15-17], it has never been used in mammals before; thus, the efficiency of using HAT-RAPD in the dog genome was analyzed. According to *Table 1*, HAT-RAPD showed a higher polymorphic ratio (16%) when compared with the use of 200 primers in conventional RAPD for identifying genetic markers associated with canine hip dysplasia (5%) [18], indicating that HAT-RAPD was suitable for producing DNA fragments in a domestic dog genome. This is in addition to its low initial investment, high speed of assay and simplicity, as described by [12,19,20].

To identify the potential candidate polymorphisms, HAT-RAPD was performed on two pooled DNA templates, patellar luxation-affected and patellar luxation-unaffected. Two polymorphic fragments from OPB05 (TGCGCCCTTC) and R105 (GCACCGAACG) primers were found (*Fig. 2*, as indicated by arrows), gel-purified, cloned and sequenced.

The results after aligning the three acquired sequences with the domestic dog *(Canis lupus familiaris)* genome in the National Center for Biotechnology Information (NCBI) database using the basic local alignment search tool (BLAST) algorithm showed that all fragments are parts of genomic scaffolds from three different chromosomes (10, 36 and X). Although none appeared to be the potentially responsible gene, these fragments can play important roles in monitoring the pathology of the disease, since an extragenic sequence can act as a promoter, enhancer, or even as an undiscovered gene, which can affect the expression of a gene or control protein functions [21]. However, when considering the flanking gene of the 659-nucleotide-long fragment located on chromosome X [KF146954], which is translated to the cAMP-dependent protein kinase catalytic subunit PRKX, the result was found to be in accordance with the findings of Wangdee et al.[22], that PL might be caused by malformation of the sartorius muscle, possibly due to the PRKX protein that functions in tissue formation, cellular differentiation and epithelial morphogenesis [23-25].

Furthermore, the 644-nucleotide-long fragment on chromosome 10 [KF146955] has one flanking uncharacterized protein [LOC403431] at 5.390 bases away on the 5' side, which can be its target of regulation since [21] stated that regulatory sequences affecting gene expression can be located both upstream and downstream of the regulatory target, with spacing up to tens of kilobases.

However, the number of chromosomes found in this study (10, 36 and X) was not consistent with chromosome 7, containing the single-nucleotide polymorphism (SNP) BICF234J1226, which has been reported to be the most significant SNP in an association analysis of 1.536 SNPs distributed over 38 autosomes and having an odds ratio of 13.6 [11]. There are 43 genes located in the 4 Mb region

surrounding the associated SNP, and none of these genes is known from other species to be involved in patellar luxation.

As a result of this study, the OPB05 primer-generated polymorphic fragment was significantly linked to patellar luxation. After alignment, the fragment was found to be genomic scaffold from chromosome 36 [KF146953] flanked by the ATP synthase gene at 398,900 bases away on the 5′ side, making the polymorphic fragment possibly linked to the gene, considering the 0.3 cM distance (less than 7 cM of linkage criteria) [26]. According to the etiology of the disorder, the patella luxates after the contraction of the quadriceps muscle. Together with the shallowness of the patellar groove, it might be possible that the luxation occurs as a result of the malfunctioning of ATP synthase, leading to the over-contraction stage of the quadriceps [27] and resulting in a floating (or luxating) patella.

Thus, HAT-RAPD has been proven to be capable of amplifying the dog genome and generating polymorphic fragments. The DNA fragment from chromosome 36 generated by the OPB05 primer (TGCGCCCTTC) showed a significant relationship with the disorder's occurrence, and is worthy of further investigation in more sample sizes with linkage analysis. In addition, further examining of three unconfirmed mutations found in the DNA fragment from the OPB05 primer should be performed. Primers specific to the region can be synthesized and used to amplify the fragment for mutation-confirmation processes, i.e. restriction enzyme cutting and sequencing. After the mutations are positively confirmed, genotyping can be performed by PCR-RFLP (restriction fragment length polymorphism) for SNP and by high-resolution polyacrylamide gel electrophoresis for deletions [28].

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