Short Communication: Ligase Detection Reaction as a genotyping technique to identify cats carrying the c.1024G>T mutation on TRPV4 gene

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Short Report

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Abstract

One of the major issues in Scottish and Highland Fold cats is osteochondrodysplasia, a bone and cartilage development disorder. A point mutation in *TRPV4* gene, associated to the ear-folded phenotype, is responsible of osteochondrodysplasia in those cat breeds. DNA amplification and genotyping by sequencing are currently the standard techniques to identify carrier animals. We developed a Ligase Detection Reaction technique, which is cheaper, more affordable and time saving analysis, obtaining 100% of correct genotyping and identification of all the fold animals as heterozygous for the c.1024G > T mutation. Ligase Detection Reaction can be used to determine the genotype of kittens even before the manifestation of the folded ears character. Despite the presence of the mutation, none of the fold animals presented clinical or radiographic signs of the disease. Thus, the association between the c.1024G > T genotype and osteochondrodysplasia, at least in young animals, is not evident. Further studies are required to fully evaluate this association.

Introduction

Scottish Fold is a purebred cat originating from Scottish Straight cat, and differs from the latter for a typical ear forward-and-downward folding feature. This feature often develops from three to four weeks of age and is usually set by the age of 3–4 months (Bell et al. 2012). The defining genetic abnormality is inherited as an autosomal incomplete dominant trait with incomplete penetrance (Takanosu et al. 2008). The first registration of a fold-eared cat occurred in 1966 and no skeletal deformities were reported until 1971, when progressive bone abnormalities and a crippling lameness were recognized (Robinson and Pedersen, 1991). Inheritance studies based on different mating schemes revealed that Scottish Fold kittens with bony lesions were only obtained from fold-to-fold mating (Jackson 1975). Because of this abnormality, Scottish and Highland Fold breeds (the latter being a long-haired variant), were banned in England in 1974 by the Governing Council of the Cat Fancy, while in the USA initial imported cats from the UK were outcrossed with several other breeds carefully avoiding fold-to-fold mating.

Osteochondrodysplasia (OCD) is quite common in Scottish Fold (~ 34%, Rorden et al. 2021). A mutant dominant allele in the *Fd* locus was considered to be the responsible of the onset of the disease, with three possible genotype-associated phenotypes: wild-type homozygous (fd/fd) with normal ears; heterozygous (Fd/fd) with folded ears and none-to-mild OCD clinical signs; homozygous (Fd/Fd) with folded ears and marked OCD clinical signs. Lesions and symptoms in homozygous cats are more severe. Thus, Fd x Fd mating is banned in some Countries. An abnormally thick tail with an inflexible base is the earliest and most consistent finding, followed by prognathia, and abnormalities in joints and bones (Malik et al. 1999). Phalanges, metacarpal and metatarsal bones are shorter and thicker than normal (Bell et al. 2012). Reduced ability to support weight, abnormal gait, stiff and stilted movements and lameness are the consequences of these skeletal degeneration. In Fd homozygous individuals, joint lesions progress until the cats are unable to walk. X-rays highlight distorted metaphyses of metatarsal and metacarpal bones and similar signs are evident in phalanges although less severe. The caudal vertebrae are shorter, endplates are widened, and gross plantar exostoses of tarsal and metatarsal bones are

clinically and radiographically evident (Zlateva and Marinov 2017). These abnormal mechanical forces are likely to end in subarticular osteoclasis and periarticular exostoses, mainly near tendons' insertions and joint capsules, linked also to degenerative joint disease and synovitis. The severe periarticular new bone formation at distal extremities may reflect the abnormal stresses to which affected joints underwent (Malik et al. 1999).

The *TRPV4* gene is located on chromosome D3, is 1740 bp long, contains 15 coding exons, and has been identified as the strongest positional candidate marker due to its mechanosensory action on chondrocytes and its response to hypotonicity. Eleven variants were detected on *TRPV4* gene sequence, and a genome wide association study revealed a significant association between fold phenotype and a missense variant (c.1024G > T, V342F) located in exon 6, confirmed by its detection only in the Scottish Fold breed after genotyping of 648 cats of several breeds of unknown ear type (Gandolfi et al. 2016). The lack of *TRPV4* gene increases bone mass in mice (Masuyama et al. 2008) and decreases rate of both mineral apposition and bone formation in human (White et al. 2016), confirming that dysregulation of chondrogenesis in bone development, skeletogenesis, and tissue patterning, leads to severe skeletal dysplasia.

Ligase Detection Reaction (LDR) is a modified Ligase Chain Reaction (LCR, first reported by Barany, 1991) that requires only two oligonucleotides as primers that bind adjacently on one target strand (Gibriel and Adel 2017). This technique is based on the biomechanism of the DNA ligase enzyme, which is highly thermostable and can be used to genotype SNPs with an excellent specificity (Zhang et al. 2021). Aim of our study was to genotype through LDR the c.1024G > T SNP in *TRPV4* gene in Straight and Fold cats and to test if this technique has better performances than the gold-standard genotyping technique.

Materials And Methods

A total of 14 cats from two Italian catteries were analyzed: 2 Highland Straight (male and female), 2 females Highland Fold, 7 females Scottish Fold, and 3 females Scottish Straight. Average age (mean ± SD) of the animals was 2.09 ± 0.85 years, ranging from 0.73 to 3.88 years. Cats were identified and examined at the University Veterinary Hospital in Grugliasco (Turin, Italy) where blood samples were contextually collected and stored in K3-EDTA blood collection tubes. Orthopedic (degree of lameness, pain, joint thickening, range-of-motion) and radiographic (orthogonal views of shoulder, elbow, hip, and stifle joints, and medio-lateral projection of the thoracic and lumbar column) examination of phenotypically Fold cats was performed by specialized hospital veterinarians to look for signs of ostechondrodystrophy and its severity. No general anesthesia nor sedation were required. DNA was extracted from blood using a DNA extraction kit (NucleoSpin Blood, Macherey Nagel, Düren, Germany) according to the manufacturer instructions. A region of 249 bp on *TRPV4* gene exon 6 was amplified using the primers TRPV4 forward and reverse (TGACAGAGAACCCGCACAA and CACTCACCCCAATCTTGCC, respectively), designed using Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). The PCR was performed in a total volume of 25 µl, using the HotStar Taq DNA Polymerase (Qiagen, Hilden, Germany). The amplification occurred at

95° for 15 min, 35 cycles of 94° for 30 sec, 54° for 30 sec, and 72° for 1 min, followed by a final extension at 72° for 10 min. Sequencing of the amplified DNA was performed for 4 samples and analyzed using the SeqStudio Genetic Analyzer (Applied Biosystems by ThermoFisher Scientific, Waltham, MA, USA).

Genotyping was performed by LDR using three probes designed in the amplified region: a common probe marked with a phosphate group and a fluorophore (TRPV4-C, Pho-TCACCAAGATGTACGACCTGCTGCTG-NED), a probe specific for the G allele (TRPV4-G, TTTTTACAACACCCGTGAGAACACCAAGTTCG), and a probe specific for the T allele (TRPV4-T, TTTTTCGCTCAGTTCGACAACACCCGTGAGAACACCAAGTTCT). The two allele-specific probes were designed adding at the 5' extremity two probe-specific queues differing in length by 10 bp, and the specific SNP allele at 3' extremity. The ligase reaction was performed in a total volume of 10 µl using the Ampligase kit (Lucigen, Wisconsin, USA) and consisted in a denaturation at 94°C for 2 min followed by 35 cycles of 90°C for 30 s, and 65°C for 2 min. The ligation mix consisted of 5 µl of PCR product, 0.2 µl of fluorescent oligomix containing 10 picomol of each probe, 0.4 µl of ligase enzyme, 1 µl of buffer, and 3.4 µl of water. Samples were subsequently analyzed by fragment analysis by capillary electrophoresis using the SeqStudio Genetic Analyzer (Applied Biosystems by ThermoFisher Scientific, Waltham, MA, USA).

Results

The DNA sequencing of the four cats revealed three mutations: c.963A > C, c.1024 T > G and c.1104 T > C (Table 1). The point mutation c.963A > C was detected in two Highland Straight (normal ears) and in one Scottish Fold (folded ears); the point mutation c.1024G > T was detected only in Scottish and Highland Fold (folded ears); the point mutation c.1104C > T was detected in one Highland Straight (normal ears) and in both Scottish and Highland Fold (folded ears).

Table 1
Genotypes by sequencing of the subset of four cats.

Animal ID	Breed	Sex	Genotypes[1]		
			c.963A > C	c.1024G > T	c.1104C > T
Cat-01	Highland Straight	Female	CC	GG	CC
Cat-02	Highland Straight	Male	CC	GG	CT
Cat-03	Highland Fold	Female	AA	GT	TT
Cat-04	Scottish Fold	Female	AC	GT	CT

[1]Alternate alleles are highlighted as underlined.

Previously described by Gandolfi et al. (2016), both c.963A > C and c.1104C > T are silent mutations and were not related to the fold phenotype in our sequenced animals. LDR was then used to genotype the

c.1024G > T mutation in all the 14 cats in our study. The obtained genotypes were consistent with the previous findings, with c.1024G > T point mutation detected only in all cats with folded phenotype.

None of the analyzed Fold animals showed clinical and radiographical alterations attributable to OCD.

Discussion

The c.1024G > T missense mutation (V342F) appeared to be associated to folded phenotype, and none of the fold cats were homozygous for the c.1024G > T point mutation, but they were all heterozygous (G/T). Studies in literature suggested that OCD in heterozygous animals has, usually, a later development in comparison to the situation in homozygous animals and shows more moderate symptoms than homozygous ones (Robinson and Pedersen 1991). Other studies reported cases of lesions radiographically evident starting from 7 weeks of age (Chang et al. 2007). This wide range of symptoms manifestations and severity fit with an incomplete dominant pattern of inheritance, with the heterozygous exhibiting incomplete penetrance and variable expressivity (Takanosu et al. 2008). Likely, other factors exert an effect on phenotypes and could explain why none of the heterozygous animals in our study, which had an average age around 2 years, showed clinical signs of the disease. This hypothesis is consistent with the results from Rorden et al. (2021), who included Scottish cats regardless of the fold phenotype and assessed the radiological data in a blind experiment, proving that the severity of the abnormalities was much less profound than shown in previously published work, and some folded-ear cats resulted similar or less severely affected than some of the straight-eared cats. Thus, it is necessary to deepen the knowledge of this disease development mechanisms in relation to the animal genotype, age, and other possible risk factors. Further follow-up studies would be required to study the evolution of the clinical signs and their association with the reported mutation and other possible mutation involved.

Ligase Chain Reaction, since its development in early ‘90s, has been recognized as an efficient tool for the discrimination of DNA strands differing for one base only (Wiedmann et al. 1994), and LDR has been used to detect DNA single nucleotides mutations (Cao 2004). In our study, we observed a perfect match between the genotypes obtained by sequencing and those obtained by LDR. Moreover, LDR is cheaper than sequencing (less than 1€/sample versus 21€/sample, respectively), and also less time-consuming since the genotyping attribution is automatable. LDR technique could be applied to genotype kittens long before the folded ears character manifests, allowing an early recognition of the animals with the alternate allele. This could be extremely useful, because of the current debate about breeding animals with this mutation for its consequences on cats’ welfare and health (Hubler et al. 2004). Thus, LDR proved itself a valuable genotyping technique, able to early identify the OCD causative mutation, long before the phenotype is expressed, and genotype crossbreed cats, where the fold phenotype cannot be observed so clearly (Takanosu and Hattori 2020).

Conclusions

LDR can be a valuable technique for genotyping single nucleotides mutation such as c.1024G > T in *TRPV4* cat gene, being cheap and easy to perform, while having the same sensitivity and specificity of the gold-standard technique. OCD mechanisms that lead to different range of severity of this condition are yet not known. Hypothetically, incomplete penetrance could be ascribed, at least partially, to the different expression of the two alleles. Being LDR a method able to quantify the ratio between alleles, its sensitivity and specificity could be exploited in further studies. Furthermore, LDR is a highly versatile technique and could be applied to other mutations, genes, and species.

Declarations

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Author contributions

Conceptualization: P. Sacchi. Genetic analysis: G. Iamone. Radiographic analysis: L.A. Piras. Data analysis: S. Chessa and R. Moretti. Writing: R. Moretti and S. Sartore. All the authors contributed to the revision of the manuscript and approved the final version of the manuscript for submission.

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Ethics approval and consent to participate

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Competing interests

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