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Missense mutation in *PFKM* associated with muscle-type phosphofructokinase deficiency in the Wachtelhund dog

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Abstract

Hereditary muscle-type phosphofructokinase (PFK) deficiency causing intermittent hemolytic anemia and exertional myopathy due to a single nonsense mutation in *PFKM* has been previously described in English Springer and American Cocker Spaniels, Whippets, and mixed breed dogs. We report here on a new missense mutation associated with PFK deficiency in Wachtelhunds.

Coding regions of the *PFKM* gene were amplified from genomic DNA and/or cDNA reverse-transcribed from RNA of EDTA blood of PFK-deficient and clinically healthy Wachtelhunds and control dogs. The amplicons were sequenced and compared to the published canine *PFKM* sequence. A point mutation (c.550C>T, in the coding sequence of *PFKM* expressed in blood) was found in all 4 affected Wachtelhunds. This missense mutation results in an amino acid substitution of arginine (Arg) to tryptophan (Trp) at position 184 of the protein expressed in blood (p.Arg184Trp). The mutation is located within an alpha-helix, and based on the SIFT analysis, this amino acid substitution is not tolerated. Amplifying the region around this mutation and digesting the PCR fragment with the restriction enzyme *MspI*, produces fragments that readily differentiate between PFK-deficient, carrier, and normal animals. Furthermore, we document 2 additional upstream *PFKM* exons expressed in canine testis but not in blood.

Despite their similar phenotypic appearance and use for hunting, Wachtelhunds and English Springer Spaniels are not thought to have common ancestors. Thus, it is not surprising that different mutations are responsible for PFK deficiency in these breeds. Knowledge of the molecular basis of PFK deficiency in Wachtelhunds provides an opportunity to screen and control the spread of this deleterious trait.

Keywords

Hemolytic anemia; Red cell defect; Myopathy; Hereditary disease; Dog

1. Introduction

Muscle-type phosphofructokinase (*PFKM*; EC 2.7.1.11), a key regulatory enzyme of anaerobic glycolysis [1,2], is expressed as a homotetramer in skeletal muscle and as a

heterotetramer in erythrocytes and various other tissues [3]. Muscle-type PFK deficiency, also known as Tarui-Layzer syndrome and glycogenosis type VII, is inherited as an autosomal recessive trait [4,5]. While PFK deficiency in humans (OMIM 232800) and horses [6] mainly promotes an exertional metabolic myopathy, the predominant clinical manifestation in affected dogs (OMIA 421-9615) is hemolytic crisis [7,8]. Several mutations of the *PFKM* gene have been described in human patients [5], but only one common nonsense mutation (c.2228G>A) has been shown to promote PFK deficiency in 3 different dog breeds [9-11]. This mutation changes a tryptophan (Trp, TGG) into a stop codon (TAG) towards the C-terminal end, truncating the last 40 amino acids (p.Trp743*) [9]. We recently described the clinical and biochemical features of PFK deficiency in Wachtelhunds [12], and here we report on its molecular basis. In addition, orthologous to the recent discovery in humans and mice of distant upstream exons of *PFKM* which are tissue-specifically spliced in testis (and embryo; also known as *TE-PFKM* [variant 1]) [13], we document the expression of a longer *PFKM* variant in canine testis but not in blood.

2. Methods

2.1. Animals and samples

The dogs studied here belong to the group of Wachtelhunds from Sweden, where the PFK deficiency was previously described [12]. Blood samples from one family of Wachtelhunds with PFK deficiency (dogs w1-w6), another affected Wachtelhund (w7), and a healthy Wachtelhund (w8) were sent to the PennGen laboratory (<http://research.vet.upenn.edu/penngen>). Leftover EDTA blood samples from dogs and one leftover canine testis following castration were also studied. The study was approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. Genetic nomenclatures used in this paper are in accordance with a recently updated version of guidelines and recommendations by the Human Genome Variation Society [14].

2.2. Genetic and PFKM sequencing studies

Genomic DNA (gDNA) and RNA were extracted from fresh EDTA blood samples from one PFK-deficient and one unrelated healthy Wachtelhund, while only gDNA was extracted from fresh or frozen EDTA blood from the other Wachtelhunds and dogs of other breeds. Genomic DNA was isolated from 200 µL EDTA-anticoagulated blood using Generation Capture Column Kit.² A column-based QIAamp RNA Blood Mini Kit³ was used for RNA extractions, blood and tissue protocols were used for fresh blood and canine testicular samples, respectively. Extracted RNA was reverse-transcribed into complementary DNA (cDNA) using High Capacity cDNA Reverse Transcription Kit.⁴

All regions of the *PFKM* gene were amplified using the Takara *Taq* HotStart⁵ and polymerase chain reaction (PCR) with primer pairs complementary to gDNA and cDNA and annealing temperatures as listed in Table 1. Human testicular *PFKM* mRNA sequence (NM_001166686.1) was used to BLAST the canine genome in order to identify its ortholog and to design the primer pair A (Table 1) that spans exons 1 to 4 of the canine *PFKM* gene. Primer pair A was used for amplification of cDNA extracted from testis and 2 different EDTA blood samples. For control purposes the same cDNA samples were also amplified using a primer pair specific to the housekeeping gene beta actin (*ACTB*). Briefly, the following conditions were applied for all PCR amplifications: each reaction was prepared to

²Qiagen, Cat.#: 159916.

³Qiagen, Cat.#: 52304.

⁴Applied Biosystems, Part #: 4368814.

⁵Takara, Cat.#: R007A.

a final volume of 50 μ L containing 2 μ L of genomic DNA or cDNA, 5 μ L of 10X PCR buffer, 4 μ L of dNTPs mixture (10 mM), and 0.5 μ L primer pairs at 10 mM concentration, 0.25 μ L of Takara *Taq* HotStart and 37.75 μ L of water. Amplification cycles used were as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles of amplification with denaturation at 95 °C for 30 s, annealing of primers at corresponding temperature for 30 s and extension at 72 °C for 30 s to 1 min, depending on the size of the amplified fragment, and a final extension at 72 °C for 7 min.

Amplified products were analyzed by electrophoresis on a 6% polyacrylamide gel and/or 1.5–2% agarose gel, then the amplicons were purified using column-based QIAquick PCR Purification Kit⁶ or QIAquick Gel Extraction Kit.⁷ Purified PCR products were sequenced at the University of Pennsylvania School of Medicine's Core DNA Sequencing Facility using the regular amplification primers. The sequencing results were assembled and compared to the *PFKM* gDNA and cDNA (NM_001003199.1) sequences on NCBI's Gene database (Gene ID: 403849) using the DNASTAR⁸ and ABI Sequence Scanner software, UCSC Genome Browser [15], NCBI-Blast [16], and Ensembl [17] web based programs. The SIFT tool [18] was used to determine the impact of the identified polymorphism. The secondary structure of the canine *PFKM* protein was assessed by using PSIPRED (a protein structure prediction server [19]) and also by comparing the canine *PFKM* protein to the rabbit *PFKM* protein structure available on the UniProt database [20].

A DNA screening test was developed, with a specific primer pair (Forward 5' - TCACAGCGGAGGAGGCTACGAA-3', Reverse 5' - AGCAGTGCAGTCTCAGGCCCTA-3') to amplify a 314 bp product of exon 8 of *PFKM*. This PCR product was then digested with the restriction enzyme *MspI*⁹ for 2 h at 37 °C, to differentiate between normal, carrier and affected Wachtelhunds.

3. Results

The coding regions of *PFKM* from healthy Wachtelhunds are identical to the published canine *PFKM* cDNA sequence (NM_001003199.1). Furthermore, compared to the canine *PFKM* cDNA from blood, analysis of the testicular *PFKM* cDNA revealed the expression of 2 additional exons of 82 and 123 bp residing about 17 kb upstream to the 5' end of the 14 bp untranslated exon of the *PFKM* cDNA in blood (NM_001003199.1). Amplification of canine testis and blood cDNA with primer pair A yielded a product only with testis cDNA, whereas the *ACTB* primer pair revealed identical products for both blood and testis cDNA (Fig. 1). Following primer pair A amplification and sequencing of *PFKM* exons 1-4 from canine testis cDNA, it is evident that 205 bp from exon 1 and 2 are expressed in testis but not in blood. Moreover, exon 3 (14 bp) is spliced out in testicular *PFKM*, but is transcribed in blood (NCBI's GenBank # JQ250035) [21]. Because of this expanded view of the canine *PFKM* gene structure, exon numbers were adjusted accordingly (Fig. 2). There may be yet another upstream exon that is untranslated in the canine testicular transcript as it is seen in human testicular transcript (NM_001166686.1).

The coding sequence from a *PFKM*-deficient Wachtelhund revealed a single base change in exon 8 (c.550C>T, based upon the *PFKM* cDNA in blood) compared to the normal sequence (Fig. 3). This missense mutation was identified by sequencing the gDNA product with the primer pair E (Table 1). The mutation results in an amino acid change from arginine

⁶Qiagen, Cat.#: 28104.

⁷Qiagen, Cat.#: 28704.

⁸DNASTAR, Madison, WI.

⁹New England Biolabs, Cat.#: R0106.

(Arg) to tryptophan (Trp) (p.Arg184Trp). The homology analysis reveals that *PFKM* is highly conserved in this region among human and animals (Fig. 4). Based upon the predicted secondary structure of the canine *PFKM* protein and the structural comparison between canine and rabbit *PFKM* proteins, the mutation is situated in one of the alpha-helix areas. Finally, SIFT analysis predicts that this amino acid substitution would not be tolerated for maintenance of enzyme function, likely due to disruption of the alpha-helix structure.

For diagnostic screening purposes, a restriction fragment length polymorphism test was developed. Digestion of the 314 bp PCR product surrounding the mutation with the restriction enzyme *MspI* results in 3 DNA fragments (147, 116 and 51 bp) for the normal (wild-type) allele. In contrast, the mutant allele is cut into 2 DNA fragments (263 and 51 bp), due to the abolishment of one of the 2 *MspI* restriction sites by the mutation (Fig. 5). In the family studied here, the one healthy parent dog available for analysis was, as expected, heterozygous for the point mutation; 3 offspring with clinical evidence of hemolytic anemia were homozygous for the mutant allele. And of the 2 healthy offspring tested, one was heterozygous and one was homozygous for the normal allele. These results are consistent with the previously reported phenotypes, based upon clinical signs and laboratory test results.

4. Discussion

A single nonsense mutation in *PFKM* has been previously described to cause PFK deficiency in English Springer Spaniels (ESSP), American Cocker Spaniels, mixed breed dogs and most recently Whippets [4,9-11]. The mutation is responsible for a terminally truncated and unstable protein [9]. As the above-cited breeds are phenotypically different but exhibited the same disease-causing mutation, a common ancestor appears likely to be responsible for transmission of the mutant allele to these breeds [10,12]. Similarly, in cases of ivermectin hypersensitivity [22] or factor VII deficiency [23,24] a single disease-causing mutation is observed in the multi-drug resistance 1 (*MDR1*) gene [25,26] and factor VII (*FVII*) gene, respectively, for several closely related breeds. Conversely, different breed-specific mutations in the same gene can cause a single disease; examples include pyruvate kinase (PK) deficiency, a common erythroenzymopathy affecting Basenjis [27], West Highland White Terriers [28], and most recently demonstrated in Labrador, Pug, Beagles and Cairn Terrier breeds [29] as well as von Willebrand disease, which is caused by several distinct mutations in the *vWF* gene in many different breeds [30]. In this report, we characterized a group of Wachtelhunds that were clinicopathologically diagnosed with PFK deficiency, but did not possess the originally described *PFKM* nonsense mutation [9,12]. Instead, disease in this breed appears to be promoted by a missense mutation in exon 8 of the *PFKM* gene.

PFK deficiency, originally described over a decade ago, was one of the first common hereditary disorders characterized at the molecular genetic level in any dog breed [9-11]. However, the mutation described here is only the second *PFKM* mutation described in dogs. In humans, 20 different *PFKM* mutations have been identified since 1990 [5,31]. Mutations have been reported in Ashkenazi Jewish, French Canadian, Swiss and Japanese patients, but genotype-phenotype associations have not been documented [5,32]. The previously described canine *PFKM* nonsense mutation and the missense mutation reported here are distinct from genetic defects reported in PFK-deficient humans [9,31,32] and thus no similarities can be deduced.

Based upon the phenotype of Wachtelhunds and their use as hunting dogs, one might have predicted that the mutation in ESSPs and Wachtelhunds would be identical. However, further research into the origin of the Wachtelhund breed reveals that they are not related to

the ESSPs. The breed was originally created from the extinct Stoberhund, which goes back several hundred years, and was founded from 11 selected Wachtelhunds throughout Germany, thereby severely limiting the breed's gene pool. The breed standard for Wachtelhunds was set accordingly at the turn of the 20th century [33,34]. Thus, it is not surprising that PFK deficiency in the Wachtelhund and ESSP breeds are caused by different mutations. The breed is still not recognized by the American Kennel Club or the Kennel Club and remains a small breed mostly represented in Germany and Sweden with a few exports to the US [35]. The last record that could be obtained from the Swedish breed club was for the year 2009, when 556 dogs were registered and 517 of them were puppies. The breed was the 26th most popular dog in Sweden in the year 2009 [33].

The frequency of *PFKM* deficiency in this hunting breed has not yet been determined. So far there are few other cases suspected in Sweden and there is no documentation of *PFKM* deficiency in Wachtelhunds from Germany or the US. Moreover, all PFK-deficient and carrier animals described in this study of Swedish Wachtelhunds can be traced to a common sire [12]. This dog was not available for study, and it remains unknown whether this sire was the first to carry the mutant allele.

The clinicopathological representation of PFK deficiency in the Wachtelhund clearly suggests protein dysfunction [12]. The Arg to Trp missense mutation (p.Arg184Trp) we describe for this breed is located in an alpha-helix area of the protein [19,20] and SIFT analysis clearly demonstrates that Arg is the only tolerated amino acid at position 184. Together, these results strongly support the role of this missense mutation in association with the disease in this breed. Future protein expression studies will be required to determine the specific dysfunction caused by this new mutation.

The development of a simple and accurate DNA test will permit the safe and conscientious breeding of carrier animals, eliminating the production of additional affected dogs and reducing any negative impact on the already limited gene pool. Breeders and breed clubs may embrace the opportunity to screen their breeding dogs to limit further transmission on both continents. However, such programs have met with variable success. Although a mutation test for PFK deficiency in ESSPs was discovered more than 2 decades ago [9], affected animals are still being diagnosed. In contrast, PK deficiency in the Basenji breed [27] seems to have been successfully eradicated with the cooperation of breeders and breed clubs (unpublished PennGen results).

The human and mouse *PFKM* gene have been extensively studied over the past decades, and the complexity of the transcriptional regulation of this critical enzyme of the glycolytic pathway has been reported. Indeed, regulation appears to be governed by multiple mechanisms, including differential splicing, use of different promoter regions, and alternative start and stop codons [13,36,37]. Recently, identification of additional exons has revealed tissue-specific splicing and expression in mouse and human testis tissues and mid-gestation mouse embryos. This alternative transcript, known as testis- and embryo-specific *PFKM* (*TE-PFKM*) or *PFKM variant 1* as per NCBI, contains additional exons that are present upstream of the previous exon 1 (which is spliced out in *TE-PFKM*). This recently identified transcript is the longest variant of the gene, however a specific function has not yet been attributed to it [13]. Notably, we were also able to document the expression of a longer *PFKM variant 1* in canine testis with 2 additional exons that are not expressed in blood. In contrast, the 14 bp untranslated exon 3 of the *PFKM* transcript in blood is spliced out in testis. These studies show the highly conserved homology in gene structure and expression in mammalian species.

In conclusion, this is the second *PFKM* mutation described in PFK-deficient dogs that causes haemolytic crises and an exertional myopathy. The breed-specific mutation test described in this study will enable the breeding of healthy mutation-free Wachtelhunds without gene pool restriction, if appropriately exploited by the breeders. Finally, this is the first report on canine testis *PFKM* variant sequence, which is tissue-specifically spliced and expressed in testicular mRNA but not in blood.

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Fig. 1.

Amplification of exons 1-4 of blood and testis cDNA samples. The upper panel represents the control ACTB gene amplifications of the same blood and testis cDNA samples, all showing similar expression of the housekeeping gene with a product length of 705 bp. Amplification of exons 1-4 of the PFKM gene in the lower panel shows no amplification in blood cDNA samples, but a 250 bp product in testis, revealing tissue-specific splicing and expression of exons 1-4. M: 100 bp marker; 1 and 2: different blood cDNA samples; 3 and 4: testis cDNA samples in duplicate; B: blank negative control.

Fig. 2.

The structure and length of the canine PFKM gene, transcripts of testis (JQ250035) and blood (NM_001003199.1) splicing variants (orthologous to human variants 1 and 4, respectively) and the breed-specific mutation sites. Exons are depicted with black vertical lines and are drawn to approximate scale, with exon numbers shown on top. The introns also are drawn to approximate scale with the exception of introns 2 and 4, which are shortened as indicated with parallel lines. The untranslated (UTR) regions in the transcripts are shown in grey color, testicular transcript has only a 3' end UTR region whereas, the blood transcript has UTR regions on both ends, on its 5' end the whole 14 bases of exon 3 and the first 8 bases in exon 4 are untranslated, the grey color of 5' UTR regions might not be distinguishable due to their smaller lengths. The Wachtelhund (*) missense and ESSP (**) nonsense mutation positions are indicated with arrows.

Fig. 3. Genomic DNA sequence chromatograms of a small region in exon 8 of the canine PFKM gene. The sequence from the healthy normal Wachtelhund is compared to that from PFK-deficient and carrier dogs. The point mutation and codon involved are indicated with an arrow and underlining.

		<div>p.Arg184Trp</div>	
		↓	
Dog	180	SALHRIIEIVDAITTT	195
Human	251	SALHRIMEIVDAITTT	266
Chimpanzee	251	SALHRIMEIVDAITTT	266
Cow	179	SALHRIIEIVDAITTT	194
Mouse	180	SALHRIVEIVDAITTT	195
Rat	180	SALHRIVEIVDAITTT	195
Chicken	169	SALHRIMEIVDAITTT	184

Fig. 4.

The amino acid sequence homology of the PFKM gene among human and animals and the site of Wachtelhund missense mutation (arrow). The conserved areas in proximity to the mutation are located within an alpha-helix and are shaded.

Fig. 5.

Pedigree and DNA restriction enzyme screening test results from PFK-deficient and asymptomatic Wachtelhund and control dogs. The uncut (lane U) band is of 314 bp length, which is cleaved at 2 sites producing 3 bands of 147, 116 and 51 bp of length for the wild-type allele (lanes: 5,8,9). The mutant allele is missing one restriction site, thus producing only 2 bands of 263 and 51 bp for the PFK-deficient animals (lanes: 1, 2, 3, 7). Samples from heterozygote animals show 4 bands (lanes: 4,6). NA: unavailable dam; M: 100 bp marker; U: uncut; 1–8 and W1–8: Wachtelhund 1–8; 9 and CTL: control dog of different breed; B: blank negative control; A: affected; C: carrier; N: normal. Open circle and square: normal female and male, respectively; black circle: affected female; half open circle and square: carrier female and male, respectively.

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Table 1

Genomic and complementary DNA primer pairs, product length and annealing temperatures used for the amplification of exons in the *PFKM* gene.

Exon amplification	Forward (5'-3') primer	Reverse (5'-3') primer	Annealing temperature	Product length
cDNA Primers				
A (Exons 1-4)	GTGTGTGATTGAGTCTCGCGGGT	ACCAGAGGTTAAACACGGCGCATGG	56 °C	250 bp ^a
G (Exons 11-17)	GTGGCTGAGGGTGGGATTGACA	GTTTGGAACCGCCTTGGCCAGT	60 °C	622 bp
H (Exons 17-21)	ATCGAGGAGGCTGGCTGGAG	CAGGCCCTCTTTCACAGTTGTTTT	60 °C	531 bp
I (Exons 20-25)	CGGATGCTGCTACATTTT	TTTTCACAGTGACCAAGTTGGCATTTA	56 °C	977 bp
gDNA Primers				
B (Exon 4)	GAGCTGACTGAGATGGCTCTTGC	ACCCAGTCCCTCCTCAAGCAGA	56 °C	228 bp
C (Exon 5)	TCCACAGATTGCCCTGTGAGGAA	CAGCATGTAACTGGGAGGTGAGGA	56 °C	400 bp
D (Exon 6)	TGTCA TGGGCATCCAGGACTCA	AGAGCAATCGTGGCACCTCTCT	56 °C	372 bp
E (Exons 7-8)	AGGAGTGACCCTGTGGTAGCTCAG	GGTGAGAAAGACGCAAGCCTACAGC	61 °C	969 bp
F (Exons 9-12)	TCTCAGGGAGCCTGCTGGAAGATC	GTGCAGTCGTGCAAGTTGTGGGTC	61 °C	1287 bp

^aPrimer pair A works only with testicular cDNA because the forward primer lies within exon 1 which is not present in blood cDNA due to tissue-specific differential splicing. Also the product length 250 bp is based on the testicular cDNA which does not include the 14 bp of exon 3 because it is spliced out in the testicular transcript.