ARTICLE

The novel homozygous *KCNJ10* c.986T > C (p.(Leu329Pro)) variant is pathogenic for the SeSAME/EAST homologue in Malinois dogs

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SeSAME/EAST syndrome is a multisystemic disorder in humans, characterised by seizures, sensorineural deafness, ataxia, developmental delay and electrolyte imbalance. It is exclusively caused by homozygous or compound heterozygous variations in the *KCNJ10* gene. Here we describe a similar syndrome in two families belonging to the Malinois dog breed, based on clinical, neurological, electrodiagnostic and histopathological examination. Genetic analysis detected a novel pathogenic *KCNJ10* c.986T > C (p.(Leu329Pro)) variant that is inherited in an autosomal recessive way. This variant has an allele frequency of 2.9% in the Belgian Malinois population, but is not found in closely related dog breeds or in dog breeds where similar symptoms have been already described. The canine phenotype is remarkably similar to humans, including ataxia and seizures. In addition, in half of the dogs clinical and electrophysiological signs of neuromyotonia were observed. Because there is currently no cure and treatment is nonspecific and unsatisfactory, this canine translational model could be used for further elucidating the genotype/phenotype correlation of this monogenic multisystem disorder and as an excellent intermediate step for drug safety testing and efficacy evaluations before initiating human studies.

INTRODUCTION

SeSAME (Seizures, Sensorineural deafness, Ataxia, Mental retardation and Electrolyte imbalance) or EAST (Epilepsy, Ataxia, Sensorineural deafness, Tubulopathy) syndrome (Phenotype MIM number 612780) is a rare, autosomal recessive multisystemic disorder.[1,2] It is caused by homozygous or compound heterozygous variations in the voltage-gated potassium channel (VGKC), inwardly rectifying, subfamily J, member 10 gene (*KCNJ10*, alias *KIR4.1*; Gene/Locus MIM number 602208). Functional KCNJ10 subunits form homotetrameric or heterotetrameric (in coassembly with KCNJ16, alias KIR5.1) channels with two putative transmembrane regions. The KCNJ10 channel is mainly expressed in the brain, spinal cord, inner ear and kidneys. Depending on their tissue localisation, these channels have distinct physiologic properties. Variations described so far have all been loss-of-function variations.[3]

The human *KCNJ10* gene is very well characterised. It is annotated in all genome browsers, has a reviewed RefSeq status (NCBI Gene ID 3766) and one transcript (CCDS1193.1; annotated by both Ensembl and HAVANA). It is located on chromosome 1q23.2 and consists of a first noncoding exon and a second exon containing the complete CDS of 1140 bp encoding a protein of 379 amino acids. Until now, 14 variants have been described causing SeSAME/EAST, all in the *KCNJ10* coding sequence (Figure 1).[4]

A similar disease (OMIA 001820-9615) has also been described in a number of dog breeds (Border collie, Dachshund and Terrier breeds; reviewed in Vanhaesebrouck *et al*[5]). Until now, only the *KCNJ10* c.627C > G (p.(Ile209Met)) variant has been reported to be associated with spinocerebellar ataxia and myokymia, seizures or both (SAMS) in certain Terrier breeds.[6,7] In addition, Forman *et al*[8] reported that the *CAPN1* c.344G > A (p.(Cys115Tyr)) variant is strongly associated with late-onset ataxia in Parson Russell Terriers, suggesting that *CAPN1* may represent a novel candidate gene for ataxia in humans. According to OMIA there are no spontaneous disease causing variants in *KCNJ10* described in other species.

Here, we describe a syndrome in two Malinois dog families with a phenotype strikingly similar to SeSAME/EAST syndrome in humans and the identification of a new pathogenic missense variant in *KCNJ10*.

MATERIALS AND METHODS

Clinical examination

Three 3-month-old intact (two males and one female) Malinois dogs (dogs 1–3) were presented at the Department of Small Animal Medicine and Clinical Biology, Faculty of Veterinary Medicine, Ghent University, for an uncoordinated gait since the age of 6 to 8 weeks. A clinical and neurological examination was performed in all three dogs, and a complete bloodwork in dogs 1 and 3. Urinalysis was performed only in dog 3. Electromyography (EMG) and brainstem auditory evoked response (BAER) tests were performed in all three dogs. EMG recordings were made from facial, truncal and appendicular muscles of front and hind limbs. Motor nerve conduction velocity studies and repetitive nerve stimulation of the radial and sciatic nerves were performed

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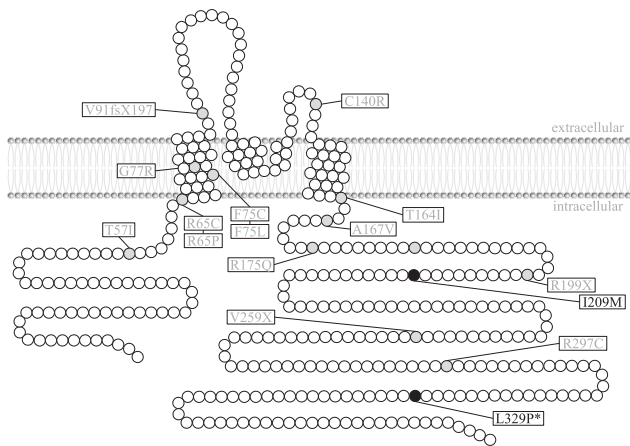
Figure 1 A predicted model of the KCNJ10 monomeric subunit (adapted from Reichold *et al*[36]), showing described SeSAME/EAST syndrome causing variants in humans (in grey)[4] and dogs (in black).[6] The KCNJ10 c.986T>C (p.(Leu329Pro)) variant described here is indicated with an asterisk.

in dogs 1 and 2. A commercially available electrophysiological unit (Medelec/TECA, Sapphire 2M, Surrey, UK) was used for electrodiagnostic recordings. EMG and BAER tests were performed under sedation and motor nerve conduction velocity studies were performed under general anaesthesia. Cerebrospinal fluid analysis and MRI (0.2 Tesla magnet) of the brain and cervical spinal cord was performed in dog 1. For affected dog 4, only video footage was available.

Pathological examination

Post-mortem examination was performed in dog 2, immediately following the killing. Central and peripheral nervous tissue samples were collected, formalin-fixed, paraffin-embedded and stained with haematoxylin–eosin or luxol fast blue. An immunohistochemical staining with neurofilament (monoclonal mouse anti-human neurofilament protein clone 2F11; Cat. No. M0762, Dako, Glostrup, Denmark) was performed.

Genetic analysis

EDTA blood samples from the four affected dogs and nine healthy family members were collected for genetic analysis (pedigree analysis in Figure 2). Genomic DNA was isolated from the blood by performing a proteinase K digestion as described in Van Poucke *et al*.[9] cDNA transcribed from canine brain RNA in Van Poucke *et al*[10] was used in this study to experimentally investigate the existence of the three predicted *KCNJ10* transcript variants (Acc. No.: X1: XM_005640901.2, X2: XM_014111261.1, X3: XM_545752.5).

Primer pairs were designed with Primer-BLAST[11] based on the canine *KCNJ10* reference sequence (Acc. No. NC_006620.3), taking into account all described transcript variants, sequence variants and repeat sequences. Primers were chosen in regions that were free of secondary structures (Mfold).[12] PCR amplicons were analysed via agarose gel electrophoresis. Sequencing reactions were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the individual PCR primers as sequencing primers and run at Eurofins Genomics (Ebersberg, Germany). Sequence analysis was performed with Unipro UGENE v1.16.1.[13] An assay with dual-labelled probes (Eurofins) was designed as described by Van Poucke *et al*[9] to genotype the *KCNJ10* c.986T>C variant in additional animals. Experimental details on the genetic analysis are given in Supplementary Information File S1A.

Bioinformatics tools for sequence variant interpretation

Variant information was gathered from population databases (Exome Aggregation Consortium[14], Exome Variant server[15], 1000 Genomes[16], dbSNP[17] and dbVAR[18]), disease databases (humsavar.txt release 2016_05[19], ClinVar[20], HGMD-public[21], OMIM[22] and OMIA[23]) and sequence databases (RefSeqGene[24], NCBI Genome[25], UCSC Genome[26] and Ensembl Genome[27]). I-Mutant2.0[28] was used as a predictor of protein stability changes upon
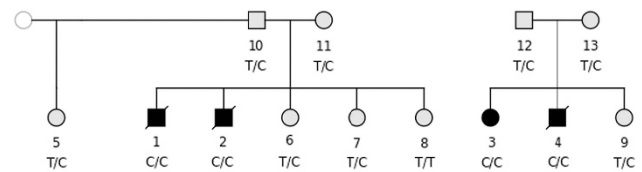

Figure 2 Sample pedigree of the 2 families (family 1 at the left and family 2 at the right) drawn with Madeline 2.0 PDE.[50] Squares are males and circles females. The white shaded icon represents a not blood-sampled dog, grey were healthy dogs and black were affected dogs. Strikethrough icons represent deceased animals. The *KCNJ10* c.986T>C genotype is shown underneath the icon.
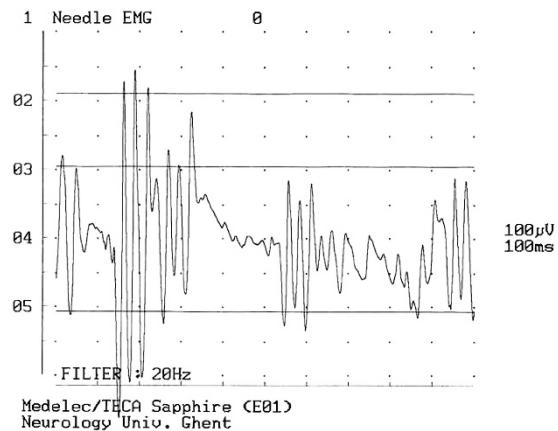

Figure 3 EMG: neuromyotonic discharges in a muscle clinically affected with myokymia. Multiple waveforms consisting of trains of 2 to 6 motor unit action potentials.

variations. Computational prediction of disease-related variants was performed with the consensus tools CONDEL[29] (combines Logre, MAPP, Massessor, Pph2 and SIFT), Meta-SNP[30] (combines PANTHER, PhD-SNP, SIFT and SNAP), PredictSNP[31] (combines MAPP, PhD-SNP, Pph1, Pph2, SIFT and SNAP) and PON-P2.[32]

RESULTS

Clinical features

General clinical examination was unremarkable in all three presented dogs. Neurological examination revealed a severe generalised hypermetric ataxia and absent patellar reflexes in all dogs (Supplementary Information File S2A). Generalised involuntary vermicular muscle contractions (myokymia) triggered by excitement were present in dogs 1 and 2 (Supplementary Information File S2B). Dog 1 had demonstrated a neuromyotonic episode of extreme generalised muscle stiffness with normal consciousness, triggered by stress. The dog also developed generalised seizures a few weeks later.

Complete blood count and serum biochemistry, including electrolytes (Na^+, K^+, Cl^-, Ca^{2+} and Mg^{2+}), was normal in dogs 1 and 3. Urinalysis (including electrolyte clearance for Na^+, K^+, Cl^-, Ca^{2+}, Mg^{2+} and PO^{4-}), was unremarkable in dog 3. EMG showed neuromyotonic discharges[5,33] in those muscles also visibly showing myokymia in dogs 1 and 2 (Figure 3), and was normal in dog 3. BAER recordings showed disappearance of wave components III/IV and V, and mildly prolonged latencies of waves I and II in dogs 1 to 3 (Figure 4). Results of motor nerve conduction velocity studies and repetitive nerve stimulation in dogs 1 and 2 did not differ from two age-matched Malinois control dogs. MRI of the brain/cervical spine and
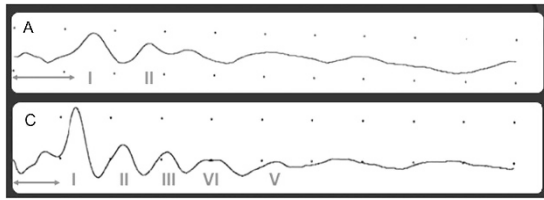
Figure 4 BAER potentials of an affected (A) and a control (C) dog (1 ms/division): loss of waves III/IV and V, prolonged latencies of all waves and prolonged interpeak latencies.

cerebrospinal fluid analysis, performed in dog 1, did not reveal any abnormalities.

The neurological condition of dogs 1, 2 and 4 deteriorated further (Supplementary Information File S2C) and all were killed because of the severity of the ataxia before the age of 6 months. Despite severely debilitating non-ambulatory ataxia, dog 3 is still alive (9 months after presentation).

Pathological features
A full necropsy was performed on dog 2 (ataxia and myokymia). No macroscopic abnormalities were found. On histopathology, bilateral myelopathy with predominant axonopathy and myelin vacuolisation was found. Changes were most obvious in the corticospinal tracts of the brainstem and the ventral funiculi of the cervical, thoracic and lumbar spinal cord. Similar lesions were found in the cerebellum (white matter) and medulla oblongata (predominantly at the pyramid bodies). In the peripheral nervous system, subtle changes were seen in a few axons (swollen with vacuolated myelin) of the brachial plexus, the femoral nerve and the sciatic nerve. Luxol fast blue and neurofilament stainings confirmed these findings (Supplementary Information File 3).

Screening for known spinocerebellar ataxia-related/causal variants
All 13 members of the 2 Malinois families (dogs 1–13, Figure 2) were first screened for the KCNJ10 c.627C>G (p.(Ile209Met)) variant (Acc. No. XM_545752.3) and the CAPN1 c.344G>A (p.(Cys115Tyr)) variant (Acc. No. XM_540866.3), as performed by Gilliam et al,[6] but none of the variants (so far only described in certain Terrier breeds) were detected. In addition, none of the variants were found in 28 healthy Malinois from different families.

Canine KCNJ10 gene characterisation
Contrary to the human KCNJ10, its canine orthologue is not yet annotated in any of the genome browsers (although assigned to chromosome 38), only has a model RefSeq status (NCBI Gene ID 488635) and three predicted transcript variants (X1-X3, with X3 being orthologous with the human transcript variant). Therefore, we first validated the predicted canine KCNJ10 transcripts experimentally. Via RT-PCR we could only confirm the presence of transcript variant X3 in the canine brain (primers 1F/1R could only amplify the 360-bp X3 fragment and not the 535-bp X1 fragment whereas primers 2F/R2 could not amplify the 140-bp X2 fragment, although they could amplify a 231-bp fragment with genomic DNA as a template; see Supplementary Information File S1A for experimental details). The other two canine transcript variants were not or very lowly (below detection limit) expressed in the canine brain, or wrongly predicted. Sequencing the complete X3 transcript showed that the canine KCNJ10 CDS was correctly predicted and has the same length compared with its human transcript orthologue, showing 90%

nucleotide sequence identity and 98% amino acid sequence identity (Supplementary Information File S1B–C).

KCNJ10 variant detection
The complete confirmed canine KCNJ10 CDS was resequenced in both parent dogs of family 1, their 5 offspring (including 2 affected dogs) and one healthy Malinois from another family. Only one variant was discovered, a c.986T>C (p.(Leu329Pro)) variant (NCBI_ss#1966650805), homozygous in the 2 affected offspring (dogs 1–2), heterozygous in both healthy parents (dogs 10–11) and 2 healthy offspring (dogs 6–7), and not present in 1 healthy offspring (dog 8) and 1 non-related healthy Malinois (Figure 2 and Supplementary Information File S1B–D).

KCNJ10 variant screening
A genotyping assay with dual-labelled probes was used to screen this variant in another healthy offspring of the father in family 1 (dog 5, heterozygous) and in family 2 (healthy heterozygous parents (dogs 12–13), 2 affected homozygous mutant offspring (dogs 3 and 4) and 1 healthy heterozygous offspring (dog 9; Figure 2). The assay was also used to estimate the frequency of this variant in 103 additional healthy Malinois from different families: 97 were homozygous wild type, 6 heterozygous and none were homozygous mutant. We also investigated whether the variant was present in the other closely related Belgian Shepherd breeds (Groenendaeler (N = 52), Laekenois (N = 28) and Tervueren (N = 56)), other Sheepdogs (Australian shepherd, German shepherd and Shetland sheepdog; all N = 25) and breeds where similar symptoms have been already described (Border collie, Dachshund, Jack Russell terrier, Maltese and Yorkshire terrier; all N = 25). However, the variant was not detected in any of these breeds.

DISCUSSION
Based on the Standards and Guidelines for the Interpretation of Sequence Variants, we conclude that the c.986T>C (p.(Leu329Pro)) variant is pathogenic for the SeSAME/EAST-like syndrome in Malinois dogs in an autosomal recessive way because of 1 strong (PS), 1–2 moderate (PM) and 3–4 (PP) supporting criteria for pathogenicity.[34]

All 4 affected Malinois dogs were homozygous mutant, whereas none of the 112 healthy Malinois dogs were. Because there were no false negatives/positives, odds ratios were infinite (PS4). Apart from the 13 members of the affected families (Figure 2), only 6 carriers were observed in the other 103 tested healthy Malinois dogs. The variant was not found in 136 dogs from 3 closely related Belgian breeds, 75 dogs from 3 other sheepdog breeds and 125 dogs from 5 breeds where similar symptoms have been already described (all dogs came from different families). In addition, a Leu was present at this position in the intracellular C-terminus in all known eutherian mammal KCNJ10 reference sequences and a variant in this codon was not found in popular population databases. A variation in this highly conserved residue is assumed to be pathogenic (PM2).

In humans, SeSAME/EAST is exclusively caused by variants in KCNJ10. Until now, 14 different pathogenic variants have been described with functional confirmation (Figure 1). A KCNJ10 missense variant was also found to segregate with a similar disease in certain Terrier breeds. Here we describe a novel KCNJ10 missense variant that segregates with the disease in two Malinois families (with two affected family members in each family; PM/PP1). As the same variation segregates in both families, they probably share a common ancestor carrying the founder variation. However, because of the lack of pedigree information, we could not identify the founder or the relatedness between both families. The allele frequency of the variant

in the Belgian Malinois population is estimated at 2.9%. Breeders are advised to screen for the variant in order to breed with variant-free animals and get variant-free offspring.

Missense variants are the most common mechanism in this disease (11/14 in humans and 2/2 in dogs) and missense variants in *KCNJ10* have a low rate of benignity (*z*-score for missense variants in *KCNJ10* is 1.78 according to the ExAC Browser, indicating intolerance to variation; PP2).

There are multiple lines of computational evidence that support the deleterious effect of this Leu → Pro variant. First, the introduction of a proline is known to affect the protein stability because of its exceptional structure. It is conformationally more rigid than any other amino acid because of its pyrrolidine ring and unlike all other amino acids it contains a secondary amide (instead of a primary amide) being unable to form a hydrogen bond.[35] According to I-Mutant2.0, the p. (Leu329Pro) variation indeed decreases stability (RI = 8). Second, according to humsavar.txt (release 2016_05) 65% of all Leu → Pro variants are disease associated, whereas the average for all variants is only 38%. Leu → Pro variants are together with Gly → Arg and Arg → Cys variants the most predominant disease-associated variants (all more than 1000 hits). In addition, all nine classified Leu → Pro variants known to exist in the KCN gene family are disease associated. Third, frequently used consensus tools for computational prediction of disease-related variants all confirm this assumption (CONDEL: 0.55, deleterious; Meta-SNP: 0.68, disease; PredictSNP: 0.55, deleterious; PON-P2: 0.91, pathogenic). Finally, the patients' phenotype and family history is highly specific for a disease with a single genetic aetiology (PP4).

The clinical features of the SeSAME/EAST syndrome are the consequence of a KCNJ10 potassium channel dysfunction.[1,36] In our four affected dogs, ataxia was always present, with or without myokymia, neuromyotonia and/or seizures. It is believed that, as in humans, the novel *KCNJ10* variant affects the function of the potassium channel in the brain and spinal cord, resulting in seizures and ataxia in the affected Malinois dogs.

Interestingly, clinical signs of myokymia and neuromyotonia (nor cramps) have not been described in humans and also not in knockout *KCNJ10* mice models. Myokymia and neuromyotonia were clinically and electrodiagnostically detected in respectively 2 and 1 of the affected Malinois dogs. The authors believe that the association between myokymia/neuromyotonia and *KCNJ10* variation is not coincidental, as another *KCNJ10* variation also caused myokymia and neuromyotonia in a large number of dogs.[6,7] In humans, myokymia and neuromyotonia have been associated with genetic and immune-mediated defects of voltage-gated potassium channels of the peripheral nerve.[37–42] However, it has not yet been associated with a potassium channel in the central nervous system. One hypothesis would be that impairment of the 'potassium sink' role by a *KCNJ10* variation not only can result in increased neuronal firing within the brain and resulting in seizures, but also in the lower motor neurons of the spinal cord, resulting in hyperexcitability of the peripheral nerve and consequently resulting myokymia and/or neuromyotonia. Another hypothesis would be that the KCNJ10 channel is also present in the peripheral nerve itself, as suggested in literature, but this has not been investigated yet. Moreover, next to direct malfunctioning of potassium channels, demyelination of the peripheral nerve has also been reported together with myokymia and neuromyotonia, another hypothesis in our animal model as demyelination of peripheral nerves was observed. The authors do not believe that systemic electrolyte disturbances would have resulted in myokymia and neuromyotonia in the affected Malinois dogs, as serum electrolytes and electrolyte clearance were normal. The fact that all dogs had ataxia, but only some dogs had seizures, myokymia or neuromyotonia, is not surprising, as it is well known that potassium channel diseases can be phenotypically heterogeneous (variability of clinical signs and severity) in dogs[33] and humans.[1,2,43]

The mildly delayed latencies of waves I and II, and the loss of waves III, IV and V, was repetitively found on the BAER tests of our affected dogs. This suggests a peripheral as well as central localisation for dysfunction within the auditory pathway.[44] This is a major difference in humans and mice, in which a cochlear dysfunction is assumed to be the origin of the hearing impairment.[1,2] The extent of hearing impairment in humans with *KCNJ10* variation is variable and can sometimes be absent or only appreciated with specific testing.[45] None of the affected dogs showed any signs of clinically relevant hearing impairment.

Another major difference with the SeSAME/EAST syndrome in humans is that the dogs in this study did not appear to show any signs of developmental delay as, apart from gait abnormalities, they behaved and were able to obey as well as their healthy siblings. Because intelligence and language testing as in humans are not applicable to dogs, we cannot exclude that a certain degree of developmental delay could have been missed in our dogs.

The histopathological findings in our dogs are identical to the ones described in the Russel Terrier group.[6,8] In knockdown mice, hypomyelination in the spinal cord with severe spongiform vacuolation, axonal swelling and degeneration is also described.[46] To the author's knowledge, there are currently no pathological reports available of humans with SeSAME/EAST syndrome. A nerve biopsy report from a human patient showed axonal neuropathy with hypomyelination, consistent with the findings in dogs.[45]

In humans, the ataxia seems to be nonprogressive and the seizures are in most cases easily controlled with antiepileptic drugs.[43] In mice, general knockout of *KIR4.1* leads to neonatal mortality.[47] In Jack Russel Terriers, the ataxia also seems to be nonprogressive, but the myokymia and neuromyotonia worsen gradually over several months, leading to killing of most dogs.[33] Because of the larger stature of Malinois dogs compared with Jack Russell Terriers, they seemed to cope less easily with the ataxia during growth of the animal. Hence, mid- to long-term prognosis in these dogs is reserved to poor.

In conclusion, we described a pathogenic c.986T > C (p.(Leu329-Pro)) variant for a SeSAME/EAST-like syndrome in Malinois dogs. Because, currently, there is no cure and treatment is nonspecific and unsatisfactory, the canine translational model could, in addition to the existing valuable model organisms such as Xenopus, mice and zebrafish that are mainly used for basic research and drug screening,[1,48] be used for further elucidating the genotype/phenotype correlation of this monogenic multisystem disorder and as an excellent intermediate step for drug safety testing and efficacy evaluations before initiating human studies.[49]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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