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A SPONTANEOUS DOG MODEL FOR A HUMAN SENSORY NEUROPATHY: IDENTIFICATION OF A MUTATION IN THE UPSTREAM REGION OF A NEUROTROPHIC FACTOR

UN MODÈLE SPONTANÉ CANIN DE NEUROPATHIE SENSORIELLE HUMAINE : IDENTIFICATION D'UNE MUTATION EN AMONT D'UN FACTEUR NEUROTROPHIQUE

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Abstract

In this study, we sought the genetic cause of self-mutilation syndrome in sporting dogs, which corresponds to human Hereditary Sensory and Autonomic Neuropathies (HSAN). We have identified a genetic mutation upstream of the gene encoding Glial cell line-Derived Neurotrophic Factor (GDNF). This mutation is responsible for insensitivity to pain in four sporting dog breeds and it perfectly segregates with the disease in 250 sporting dogs of known clinical status. Moreover, it was not found in any of the 900 unaffected dogs from 130 different breeds. Since this mutation is localized in a long non-coding RNA, we performed an in-depth analysis of the genomic region (locus) as well as gene expression analyses to understand its role in the pathophysiology of the disease. Thus, in addition to the discovery of a novel candidate gene for HSAN in humans, we propose a transcriptional regulation mechanism based on a "partnership" between GDNF and a long non-coding RNA (lncRNA).

Key words: sensory neuropathy, HSAN, pain, dog model, genetics, ncRNA.

Résumé

Dans cette étude, nous avons recherché la cause génétique du syndrome d'automutilation chez les chiens de chasse, qui correspond chez l'Homme à une neuropathie sensitive de type HSAN. Nous avons identifié une mutation en amont d'un gène codant un facteur neurotrophique : GDNF (« *Glial cell line-Derived Neurotrophic Factor* »). Cette mutation ségrége parfaitement avec la maladie chez 250 chiens de chasse de statut clinique connu et est absente chez 900 chiens indemnes de 130 autres races. Cette mutation étant située dans un ARN long non codant, une analyse fine de la région chromosomique et des études d'expression de gènes ont été réalisées pour comprendre son rôle dans la physiopathologie de la maladie. Ainsi, non seulement, nous apportons un nouveau gène candidat pour les neuropathies humaines, mais nous proposons également un mécanisme de régulation transcriptionnelle original, basé sur un « partenariat » entre le gène codant le facteur neurotrophique et l'ARN long non codant.

Mots-clés : neuropathies sensorielles, HSAN, douleur, modèle chien, génétique, éléments régulateurs.

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INTRODUCTION

Human inherited peripheral neuropathies, including Charcot-Marie-Tooth diseases (CMT) and hereditary sensory and autonomic neuropathies (HSANs), are neurodegenerative diseases (Jerath & Shy, 2015). The main symptom is a progressive degeneration of sensory and autonomic neurons, causing insensitivity to pain and temperature in feet and hands, sometimes leading to ulcerative mutilations (Indo, 2014). To date, 12 genes have been associated with human HSANs, however they only explain one third of affected patients. Therefore, spontaneous models of these diseases are needed to find new implicated genes. Interestingly, due to breeding practices, some dog breeds have spontaneously developed such diseases with high frequencies (Granger, 2011). Four sporting breeds (German Shorthaired Pointers (GP), English Pointers (EP), English Springer Spaniel (ESS) and French Spaniel (FS)) are affected with diseases manifesting symptoms comparable to human HSANs (Paradis *et al.* 2005). In the affected dogs, the hallmarks of the disease are loss of pain sensation and feet ulceration, with the age of disease onset around four months old. Symptoms lead, in the majority of cases, to severe self-mutilations of the feet including claw loss, painless fractures, and digit amputations ***(figure 1)***. In this study, a large pedigree of French spaniels was first drafted; careful analysis of this pedigree revealed a recessive autosomal mode of inheritance, as previously described (Paradis *et al.* 2005). A genetic association study has been performed and revealed a locus that was highly associated with the disease, on canine chromosome 4. Re-sequencing of the locus led to the identification of a strongly associated variant within the regulatory region of *GDNF* (*Glial cell line-Derived Neurotrophic Factor*), a neutrotrophic factor.

MATERIALS AND METHODS

Ethics statement and sample collection

The work with dog samples was approved by the CNRS ethical board, France for UMR6290. The biological samples were obtained from the 'Cani-DNA CRB', (http://dog-genetics.genouest.org) from veterinarians in the course of the dog follow-up and the dog owners consented to the use of data for research purposes anonymously.

Nucleic acid extraction

Genomic DNA was extracted from EDTA blood samples, using the NucleoSpin® Blood L kit (Macherey-Nagel). RNA was extracted from Dorsal Root Ganglia (L6 and L7), using the NucleoSpin® RNA kit (Macherey-Nagel).

Genome-Wide Association Study (GWAS), locus sequencing by Next-Generation Sequencing (NGS) and Allele-Specific PCR

Using the Illumina Canine HD 173k (BeadChip), 62 dogs were genotyped at the Centre National de Génotypage (CNG; Evry, France) and statistical analyses were performed using Plink software (v1.06-1.07) and GEMMA software (v0.94.1) (Purcell *et al.* 2007; Zhou & Stephens, 2014). Locus sequencing was performed by capture-sequencing on four affected and four unaffected dogs by Integragen (Evry, France): the genomic libraries were made using the Illumina paired-end library sample preparation kit (Illumina Inc.), the capture experiment was performed with the Agilent SureSelect Target Enrichment System Kit and samples were paired-end sequenced on an Illumina HiSeq2000. Variants were identified using GATK software (McKenna et al, 2010) and candidate variants were genotyped on DNA from 400 dogs from 100 breeds, using an Allele Specific PCR method (AS-PCR) (Integragen).

Gene expression analysis

Reverse transcription was done on total RNA using the High-capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific). Pre-amplification was performed on cDNA with the TaqMan PreAmp Master Mix Kit (Thermo Fisher Scientific). qRT-PCR was performed on diluted pre-amplified cDNA with the TaqMan PCR master mix (Thermo Fisher Scientific) on the 7900HT Fast Real-Time PCR System (Applied Biosystems) using standard procedures. Each qRT-PCR was carried out in triplicate using pre-designed primers of *GDNF* (Thermo Fisher Scientific Reference: Cf03986046_g1), *WDR70* (TFS Ref: Cf02651565_m1), *NUP155* (TFS Ref: Cf02644933_m1) and SLC1A3 (TFS Ref: Cf02702629_m1). We also specifically designed other probes for *GDNF-AS* as well as the canine PPIB (*Peptidylprolyl Isomerase B*) gene for reference (Thermo Fisher Scientific). Relative amounts of the transcripts were determined using the standard procedures of the ΔΔCt method.

RESULTS

Identification of the mutation

We focused the genetic analysis on the French Spaniel breed, using the DNA of 173 dogs with pedigree and clinical information. The affected dogs presented severe self-mutilations with insensitivity to pain in the feet ***(figure 1)***. A genome-wide association study (GWAS) with 49 French Spaniels (21 affected and 28 unaffected dogs) led to the identification of a 3 Mb locus on canine chromosome 4 (CFA4) strongly associated with the disease ***(figure 2)***. Whole genome genotyping was then performed on dogs from the three other affected breeds, revealing a common homozygous haplotype, allowing us to narrow down the critical interval to 1.8 Mb.

Targeted re-sequencing of this locus of interest was performed on four affected and four unaffected sporting dogs. After quality controls and filtering of known canine polymorphisms (SNPs), 156 variants remained. Using Allele Specific-PCR (AS-PCR), only one SNP perfectly segregated with the disease. Using Sanger sequencing in 250 sporting dogs of known clinical status, we

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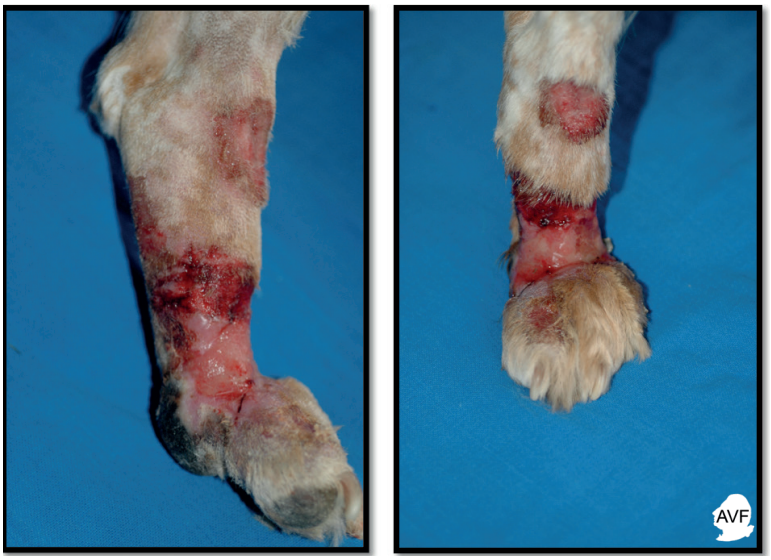

Figure 1 : *Ulceration and fibrosis on a podale end of a dog affected with sensory neuropathy. Photos by Éric Guaguere.*

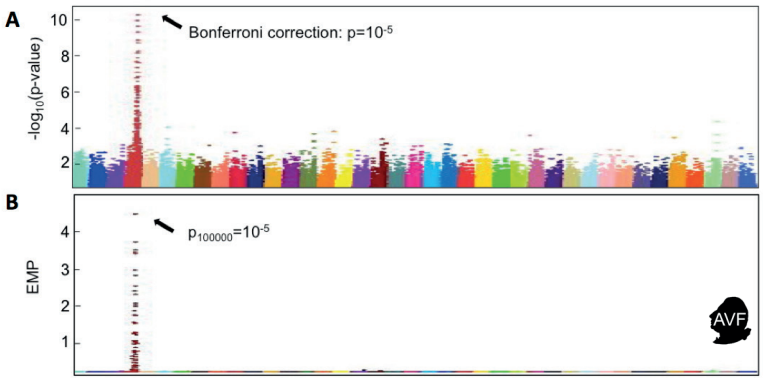

Figure 2 : *(A) Manhattan plot of -log10 of p-values by chromosome. We identified a strong signal on chromosome 4 with Bonferroni corrected p-values ≤ 10-5. (B) Manhattan plot of -log10 empirical p-values (EMP) obtained by permutations test confirmed the signal on chromosome 4 (Permutation=100,000).*

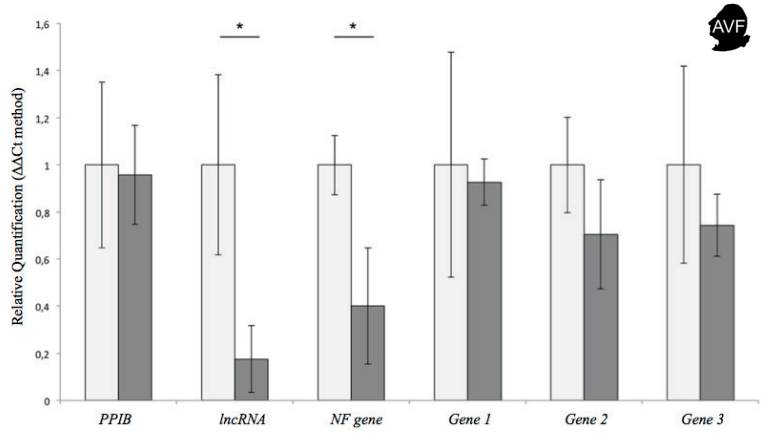

Figure 3 : *qRT-PCR results in Dorsal Root Ganglia using the ΔΔCt methodology (cases in grey, controls in white). PPIB gene was used as a control and levels of all genes and lncRNA were tested 5 times with 3 replicates for each (t-test: p-value *<0.0001). The lncRNA, GDNF (denoted as NF gene), and their neighboring genes (WDR70, NUP155 and SLC1A3 noted Gene 1, Gene 2 and Gene 3 respectively) are ordered as in the canine genome (CanFam3 assembly).*

observed that the alleles of this SNP followed the expected transmission mode. In addition, the mutated allele was absent among 900 unaffected dogs from 130 different breeds.

Genomic analyses

The variant (chr4.g.70,875,561C>T) is located in an intergenic region, 90 kb upstream of *GDNF*, known to be involved in the regulation of sensory neuron (Buj-Bello *et al.* 1995). Using an "in-house" improved annotation of the CanFam 3 genome assembly, the presence of a long non-coding RNA (lncRNA) was detected in the vicinity of *GDNF* (Wucher *et al.* 2017). Interestingly, the variant is located in the last exon of this lncRNA, potentially regulating *GDNF*.

Expression analyses by qRT-PCR

To test our hypothesis that the variant affects the expression of *GDNF*, and thus better understand its effect on the disease, we measured expression levels of both *GDNF* and the lncRNA. As *GDNF* is known to be expressed in Dorsal Root Ganglia (DRG) (Molliver *et al.* 1997), we measured the expression levels of *GDNF* and the lncRNA in DRG. Quantitative real-time PCR revealed that the levels of *GDNF* mRNA and lncRNA expression were both significantly decreased in affected dogs (60% and 80% respectively, p<0.0001). We also showed that the expression levels of the other genes located in the locus were not altered ***(figure 3)***, indicating that the mutation impacts only *GDNF* and the lncRNA. Thus we demonstrated that *GDNF* and its lncRNA have a correlated expression that is affected by the mutation.

DISCUSSION

The long-term collaboration between our research team and veterinarians involved in genetic diseases allowed the identification of a mutation responsible for self-mutilation in sporting dogs and introduces a new candidate gene for human sensory neuropathies. The project focused on dogs with self-mutilations as determined by a precise clinical questionnaire and allowed us to detect that all dogs with self-mutilations also presented insensitivity to pain, a symptom not always reported by the owners. We identified additional dogs presenting the "affected" homozygous haplotype, but without self-mutilation. Indeed, their insensitivity to pain was not primarily detected, but the owners reported an abnormal gait. This observation reflects the difficulty in diagnosing such neuropathies in dogs,

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presenting symptoms from insensitivity to pain to self-mutilation. This situation contributed to the spreading of this severe disorder in the four related sporting breeds (Paradis *et al.* 2005). This feature led us to consider self-mutilation, probably triggered by small fractures of toes or other injuries, as a consequence of the insensitivity to pain.

We collected DRG samples since DRG neurons and peripheral nerves were previously shown to be altered in affected dogs (Cummings *et al.* 1983). The qRT-PCR analysis of canine DRG led to the important finding that the mutation seems to affect a regulatory element of *GDNF*. Indeed, while the mutation is located in the last exon of the lncRNA, the expression levels of both *GDNF* and the lncRNA are decreased in affected dogs compared to unaffected dogs ***(figure 3)***. Thus, the mutation impacts the fine-tuning of *GDNF* and its lncRNA expression levels. Our data support the implication of *GDNF* in the disease, and suggest that insufficient levels of this factor in the peripheral nervous system could prevent the growth and maintenance of the sensory neurons in the paws of affected puppies.

CONCLUSION

We identified the mutation responsible for a sensory neuropathy in sporting dogs, located in the last exon of a lncRNA and upstream of the neurotrophic factor *GDNF*. We showed that the lncRNA is co-regulated with *GDNF* and that the mutation dramatically diminishes both the expression of *GDNF* and the lncRNA. We anticipate that the disease is due to a decrease of *GDNF* mRNA expression, preventing the correct growth and maintenance of the small nerve fibers of the paws. To fully demonstrate this, we aim to perform electron microscopy on the sensory neurons of affected dogs. The identification of this mutation has led to the development of a genetic test useful for diagnosis and breeding to decrease disease frequency in predisposed breeds.

In addition, *GDNF* is a relevant candidate for human HSAN and in pain mechanisms generally. We are therefore screening this candidate gene in affected human patients since only one third of HSAN patients are explained by known mutations.

Our work illustrates the power of the canine model in genetics and also reveals the importance of exploring regulatory elements proximal to coding genes in human and dog diseases. Searching for the genetic causes of other neuropathies in dog breeds manifesting high incidence, has the potential to uncover novel genes important for both human and canine medicine.

A more detailed description and analysis of the cellular impact of the mutation herein described is published in Plassais *et al.* 2016.

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