A de novo mutation in the EXT2 gene associated with osteochondromatosis in a litter of American Staffordshire Terriers

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Background: We aimed to identify mutations associated with osteochondromatosis in a litter of American Staffordshire Terrier puppies.

Hypothesis: We hypothesized that the associated mutation would be located in a gene that causes osteochondromatosis in humans.

Animals: A litter of 9 American Staffordshire puppies, their sire and dam, 3 of 4 grandparents, 26 healthy unrelated American Staffordshire Terriers, and 154 dogs of 27 different breeds.

Methods: Whole genome sequencing was performed on the proband, and variants were compared against polymorphisms derived from 154 additional dogs across 27 breeds, as well as single nucleotide polymorphism database 146. One variant was selected for follow-up sequencing. Parentage and genetic mosaicism were evaluated across the litter.

Results: We found 56,301 genetic variants unique to the proband. Eleven variants were located in or near the gene exostosin 2 (EXT2), which is strongly associated with osteochondromatosis in humans. One heterozygous variant (c.969C > A) is predicted to result in a stop codon in exon 5 of the gene. Sanger sequencing identified the identical mutation in all affected offspring. The mutation was absent in the unaffected offspring, both parents, all available grandparents, and 26 healthy unrelated American Staffordshire Terriers.

Conclusions and Clinical Importance: These findings represent the first reported mutation associated with osteochondromatosis in dogs. Because this mutation arose de novo, the identical mutation is unlikely to be the cause of osteochondromatosis in other dogs. However, de novo mutations in EXT2 are common in humans with osteochondromatosis, and by extension, it is possible that dogs with osteochondromatosis could be identified by sequencing the entire EXT2 gene.

Keywords
bone, cartilage, genetics, multiple cartilaginous exostoses, mutation, osteochondroma

Abbreviations: ADS, Amplicon-based deep sequencing; EXT1, exostosin 1; EXT2, exostosin 2; GATK, Genome Analysis Toolkit; LOH, loss of heterozygosity; NCSU, North Carolina State University; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism; UMGC, University of Minnesota Genomics Center; VQSR, variant quality score recalibration; WGS, whole genome sequencing.

This work was performed at the Veterinary Genetics Laboratory at North Carolina State University and the Canine Genetics Laboratory at the University of Minnesota.

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1 | INTRODUCTION

Osteochondromatosis a rare skeletal developmental disorder characterized by the appearance of cartilage capped boney outgrowths (exostoses) that continue to develop and grow until skeletal maturity. Exostoses arise from the periosteum of bones that undergo endochondral ossification, frequently but not exclusively near physes. Common sites include the long bones as well as the vertebrae and ribs. Solitary lesions are known as osteochondromas whereas multiple lesions are referred to as osteochondromatosis, or multiple cartilaginous exostoses. Clinical signs may occur as a result of compression of adjacent tissue, for example, pain caused by nerve compression, or paralysis associated with spinal cord compression. In <5% of cases, the benign lesion can undergo malignant transformation.

The condition has been reported in people, horses, dogs, and cats, although the condition in cats appears to differ from that in other species, occurring after skeletal maturity. It is inherited as an autosomal dominant trait in people, with >90% of cases being associated with a loss of function mutation in exostosin 1 or 2 (EXT1 or EXT2). Both of these genes encode glycosyltransferases important in the generation heparan sulfate. Although most reports in dogs describe solitary cases, the occurrence of osteochondromatosis in littermates and successive generations of dogs supports a hereditary etiology in this species, but an associated genetic variant has not yet been described.

The purpose of our report is to describe the results of an investigation into the genetic cause of osteochondromatosis in a litter of American Staffordshire terriers containing 3 affected dogs. The clinical features, results of whole genome sequencing (WGS), and follow-up Sanger sequencing of variants in a wider population of both related and unrelated dogs is reported.

2 | MATERIALS AND METHODS

2.1 | Animal selection and phenotyping

The proband was presented to the Neurology Service at North Carolina State University (NCSU) College of Veterinary Medicine for evaluation of progressive paraparesis. Examination of the proband included physical and neurological examinations, routine CBC, and serum biochemistry panel, and magnetic resonance imaging (MRI; 1.5T magnet, Symphony, Siemens, Cary, North Carolina) of the thoracolumbar spine. After euthanasia, a full necropsy was performed. The dog’s breeder provided information on the full litter, and 2 littermates showing paraparesis were evaluated by board-certified veterinary neurologists at other institutions. One dog had spinal radiographs performed and both dogs underwent necropsy. Blood samples were collected from the entire litter (affected and unaffected dogs), both parents, 3 of 4 grandparents, and 26 other unrelated American Staffordshire terriers evaluated for various non-neurologic diseases at NCSU.

2.2 | DNA isolation

Approximately 2–3 mL of ethylene diamino tetra-acetic acid (EDTA)-anticoagulated blood was collected from each animal and shipped to the Veterinary Genetics Laboratory at North Carolina State University. The DNA was extracted using the standard protocol of the DNeasy Blood and Tissue Kit (Qiagen). Quality and concentration of DNA were evaluated using both a spectrophotometer (Nanodrop 1000) and a fluorometer (Qubit 2.0).

2.3 | Whole genome sequencing

Approximately 3 μg of DNA from the proband was submitted for Illumina TruSeq PCR-free library preparation and WGS at Genewiz (South Plainfield, New Jersey). Sequencing experiments were designed as 150 base pair (bp) paired-end reads and the library was run in 1 lane of an Illumina HiSeq 4000 high-throughput sequencing system. These reads have been made publicly available at the NIH Short Read Archive (https://www.ncbi.nlm.nih.gov/sra/SRP126770).

Variant calling from WGS data was performed using a standardized bioinformatics pipeline as described previously. Briefly, sequence reads were trimmed using Trimmomatic 0.32 to a minimum phred-scaled base quality score of 30 at the start and end of each read with a minimum read length of 70 bp, and aligned to the University of California at Santa Cruz canFam3 reference sequence using Burrows-Wheeler Aligner (BWA) 0.7.13. Aligned reads were prepared for analysis using Picard Tools 2.8 (http://broadinstitute.github.io/picard) and Genome Analysis Toolkit (GATK) 3.7 following best practices for base quality score recalibration and indel realignment specified by the Broad Institute, Cambridge, Massachusetts. Variant calls were made using GATK’s HaplotyveCaller walker, and variant quality score recalibration (VQSR) was performed using sites from single nucleotide polymorphism database 146 and the Illumina 174K CanineHD BeadChip as training resources. We applied a VQSR tranche sensitivity cutoff of 99.9% to single nucleotide polymorphisms (SNPs) and 99% to indels for use in downstream analyses; genotype calls with a phred-scaled quality score < 20 were flagged but not removed from the variant callset.

2.4 | Variant filtering and annotation

Variants (either heterozygous or homozygous) present in the proband were filtered against a database of whole genome sequences derived from 154 dogs that have been sampled as part of ongoing research in our laboratory and the laboratories of our collaborators. These include 22 Boxers, 20 Standard Poodles, 13 Great Danes, 13 Yorkshire Terriers, 11 Cavalier King Charles Spaniels, 9 Dachshunds, 8 Scottish Deerhounds, 6 Scottish Terriers, 6 Miniature Poodles, 4 Doberman Pinschers, and 42 dogs of 18 additional breeds. Sequence data from all of these dogs were processed using the same bioinformatics pipeline described above. None of the animals in this database were known to have osteochondromatosis or any other similar bone or cartilage disorder. Variants that passed our filtering step were annotated using SnpEff 4.3.

2.5 | Sanger sequencing

Based upon our WGS findings, we performed Sanger sequencing to evaluate 1 SNP in exon 5 of exostosin glycosyltransferase 2 (exostosin 2) in all 9 offspring, both parents, 3 of 4 grandparents (DNA from 1 of the
grandparents was unavailable for testing), and 26 unrelated breed-matched dogs. A 337 bp region flanking the SNP of interest was amplified using a polymerase chain reaction (PCR) under standard reaction conditions with the following primers: F-5′-TCTGCACAACGGTACA CC-3′ and R-5′-ACAGAGAAGACCTGGAAGCC-3′. The resulting PCR amplicon was sequenced by Sanger sequencing on an Applied Biosystems 3730xl DNA sequencer at the University of Minnesota Genomics Center (UMGC).

2.6 | Parentage testing

Parentage testing was performed on all offspring and both parents using a commercially available microsatellite-based marker test performed at the University of California Davis Veterinary Genetics Laboratory (https://www.vgl.ucdavis.edu/services/CatandDogDNATyping.php).

2.7 | Amplicon-based deep sequencing

Amplicon-based deep sequencing (ADS) was performed on both parents and all offspring to test for genetic mosaicism that could have given rise to a heritable or somatic mutation in EXT2. This technique provides extremely high coverage (>×1000) of a particular amplicon so that low-copy-number heterozygotes, which could indicate genetic mosaicism and would ordinarily not be detectable by Sanger sequencing, can be identified. Standard Illumina Nextera sequencing primers were appended to the 5′ end of the EXT2 primers described above, and PCR was carried out for each dog to generate a 337 bp amplicon with 5′ and 3′ tails suitable for barcoding. The PCR amplicons were submitted for Illumina i5 and i7 barcoding, multiplexing, library preparation, and sequencing at UMGC.

All 11 amplicons were pooled in equimolar amounts and sequenced on an Illumina MiSeq Desktop sequencer. Reactions were designed as 2×300 bp paired-end reads using Illumina v3 chemistry. De-multiplexed sequencing reads were processed using the same bioinformatics pipeline as described above, but VQSR was not performed given the small target region.

3 | RESULTS

3.1 | Animal phenotyping

The proband, a male American Staffordshire Terrier, presented at 4 months of age with progressive paraparesis first noticed 2 weeks previously as weakness and dragging of the right hind limb. The dog’s weakness progressed rapidly despite treatment with an anti-inflammatory dose of prednisone and clindamycin (for suspected Neospora caninum infection). At the time of presentation, the dog was non-ambulatory paraparetic, worse on the right hind limb. General physical examination was unremarkable. The neuroanatomic localization was the third thoracic (T3) to third lumbar (L3) spinal cord segments based on paraparesis with normal patellar and withdrawal reflexes. Routine laboratory tests did not show any clinically relevant abnormalities, and inspection of radiographs obtained 2 weeks previously by the referring veterinarian disclosed a bony growth on the right 13th rib but no other abnormalities. Magnetic resonance imaging identified large bony outgrowths from the dorsal lamina of the first lumbar vertebra (L1) compressing the spinal cord. (B) Transverse proton density image at the level of L1 showing 2 exostoses (wide arrows), one originating in the dorsal lamina of L1 and the other on the right 13th rib. The spinal cord is compressed into the left ventrolateral aspect of the vertebral canal (thin arrow) by the vertebral lesion. (C) Transverse T1-weighted image at the level of L1 after contrast administration. The ventral rim of the L1 lesion contrast enhances (arrow).
compressive lesion at C8. This pup also had 2 rib lesions. A third littermate, a female, developed paraparesis around 7 months of age. The neuroanatomic localization again was T3-L3 spinal cord segments and spinal radiographs disclosed multiple vertebral lesions (Figure 2). The puppy was euthanized and the diagnosis confirmed at necropsy. The expansile masses affecting ribs and vertebrae were composed of an outer layer of hyaline cartilage undergoing orderly endochondral ossification to bony trabeculae, with intertrabecular hematopoietic tissue (Figure 3).

The remaining littermates grew to skeletal maturity without developing any clinical signs and have remained neurologically normal. The sire and dam were neurologically normal with no history of similar problems in their pedigrees. The dam (5.75-year-old American Staffordshire Terrier) had produced 3 healthy litters (26 offspring in total) with 3 other sires before this litter, and the sire (1-year-old American Staffordshire Terrier) had only been bred once before to a different dam. None of these offspring had developed osteochondromatosis to the breeder’s knowledge.

3.2 | Whole genome sequencing

The WGS of the proband identified 56,301 variants representing 67,379 variant effects that were unique to the dog. Four intronic variants were located in EXT1 and 11 variants were located in or near EXT2 (Table 1). Among the variants in EXT2, 1 heterozygous variant in exon 5 of the gene (Ensembl Transcript ID ENSTCAFT00000150565.3, c.969C > A, genomic coordinates chr18:45101754) is predicted by SnpEff to introduce a stop codon (p.Tyr323Ter). There were 62 other variant effects across the entire genome of the proband that were classified by SnpEff as having a high impact on gene function, and 247 variant effects classified as having a moderate impact on gene function. None of these variants were located in any gene known to be associated with osteochondromatosis in humans.

3.3 | Sanger sequencing

Sequencing of the proband confirmed a heterozygous variant at chr18:45101754. This same heterozygous variant was noted in the 2 other affected littermates, but not in any of the unaffected littermates, the parents, the grandparents from which DNA was available, or any of the other healthy unrelated American Staffordshire Terriers (Figure 4). These findings suggested a possible de novo mutation in the affected offspring that was unique to this litter.

### Table 1 | Variants and variant effects in EXT1 and EXT2 unique to the proband

| Gene | Chr | Position | Variant | Predicted effect |
|------|-----|----------|---------|-----------------|
| EXT1 | 13  | 17,346,529 | c.956−79370A>G | Intron |
| EXT1 | 13  | 17,346,529 | c.962 + 108979A>G | Intron |
| EXT1 | 13  | 17,413,735 | c.955 + 41780A>C | Intron |
| EXT1 | 13  | 17,413,735 | c.962 + 41773A>C | Intron |
| EXT2 | 18  | 44,979,553 | G>A | Downstream |
| EXT2 | 18  | 44,979,638 | G>T | Downstream |
| EXT2 | 18  | 44,979,767 | A>G | Downstream |
| EXT2 | 18  | 44,979,807 | A>G | Downstream |
| EXT2 | 18  | 44,979,942−44,979,943 | insA | Downstream |
| EXT2 | 18  | 44,980,454 | C>T | Downstream |
| EXT2 | 18  | 44,987,913 | c.*14C>T | 3’ Untranslated region |
| EXT2 | 18  | 44,999,840 | c.1708−588C>T | Intron |
| EXT2 | 18  | 45,006,007 | c.1708−6755C>T | Intron |
| EXT2 | 18  | 45,101,754 | c.969C>A | Stop gained |
| EXT2 | 18  | 45,105,165 | c.789−3231C>T | Intron |

EXT1, exostosin 1; EXT2, exostosin 2.

The highlighted row represents the putative causative mutation for osteochondromatosis in the proband and its littermates. Note that each variant in EXT1 has 2 variant effects based upon different predicted annotations for that gene.
3.4 | Parentage testing

Parentage was confirmed for all offspring (>99% confidence for each test), indicating that no mismating occurred that could have introduced the identified EXT2 mutation into the affected offspring.

3.5 | Amplicon-based deep sequencing

Depth of coverage from ADS across all dogs sequenced ranged from 8800 to 901,940 x. For both parents and all unaffected offspring, >99.8% of the reads showed a C (reference) allele at the EXT2 exon 5 mutation site. For the affected offspring, the reads were equally balanced between C and A alleles, ranging from 48.8% to 53.7% of reads for the C allele.

4 | DISCUSSION

Our findings provide compelling evidence that a single copy of a de novo mutation in exon 5 of the EXT2 gene is associated with the development of osteochondromatosis in this litter of American Staffordshire Terriers. This heterozygous mutation was uniquely present in all affected littermates, yet absent in all family members available for testing, a population of unaffected, unrelated American Staffordshire terriers, a population of unrelated dogs of other breeds, and the most recently available public canine SNP database. This mutation also is predicted to introduce a stop codon in a protein-coding portion of a gene that has been strongly implicated in osteochondromatosis in humans.2,7,21

**Figure 4** Sanger sequencing results for the exostosin 2 (EXT2) mutation associated with osteochondromatosis in this litter. (A) Chromatograms showing a heterozygous mutation in 3 littermates with osteochondromatosis (bottom 3 lines) and the reference genotype in both parents; the mutation site is highlighted. (B) Three-generation pedigree with genotypes for the EXT2 mutation. Affected dogs are colored red; genotypes are shown below each animal’s pedigree symbol; the proband is noted with an arrow.
In humans, osteochondromatosis, also referred to as multiple cartilaginous exostoses or hereditary multiple exostoses, is a relatively rare condition that is strongly associated with mutations in the genes EXT1 or EXT2. Mutations in these genes have been described in between 70 and 94% of patients with osteochondromatosis, with about 2/3 of the mutations being in EXT1. However, this proportion can vary widely by population subgroups. An international mutation database for osteochondromatosis in humans has documented 432 unique variants in EXT1, and 223 unique variants in EXT2 that have been associated with the disease. Most of these presumptive deleterious variants are nonsense, frameshift, or splice site variants that are predicted to result in loss of or diminished protein function. In the majority of inherited cases, the condition is dominantly inherited, such that only 1 defective allele copy is necessary to trigger the disease. Approximately 10% of human patients have a de novo mutation, as we have documented in this litter of dogs.

One study of osteochondromatosis mutations in humans suggested that somatic mutations or genetic mosaicism may be responsible for osteochondromatosis in the small number of cases for which a germline variant in EXT1 or EXT2 cannot be identified. Because Sanger sequencing of both parents failed to identify the EXT2 mutation we identified in the 3 affected offspring, we considered this possibility in the affected dogs presented here. However, ADS definitively ruled out genetic mosaicism or any low-level somatic mutation, leading us to conclude that the mutation arose de novo across the litter. We unfortunately were unable to obtain gonadal tissue from either the sire or the dam of this litter to confirm this possibility, however de novo mutations are a widely recognized source of genetic mutations responsible for an array of congenital disorders, including osteochondromatosis in humans.

The exostosin family of genes encodes glycosyltransferases that are responsible for the synthesis and assembly of heparan sulfate chains on proteoglycans. Both heparan sulfate and proteoglycans are critically important for normal bone and cartilage development. Because both EXT1 and EXT2 are required for heparan sulfate synthesis, heterozygous mutations in either gene results in systemic deficiency of these molecules in approximately 50% of patients with osteochondromatosis. The exact mechanism by which mutations in EXT1 and EXT2 cause osteochondromatosis is unknown, however 2 theories have been advanced: haploinsufficiency and loss-of-heterozygosity (LOH). The first model suggests that the lower gene dosage associated with a heterozygous mutation causes insufficient amounts of heparan sulfate, and results in an environment in which osteochondromas can proliferate. The LOH model suggests that EXT1 and EXT2 function as tumor suppressor genes, and that loss of function of 1 copy of these genes is followed by a second mutation during a somatic mutation event, which inactivates the tumor suppressor gene and results in the formation of osteochondromas. Although fully elucidating the specific mechanism by which the EXT2 mutation identified here caused osteochondromatosis in this litter of dogs is beyond the scope of our study, mechanistic studies in humans related to the role that the exostosin genes play in causing osteochondromatosis lay the groundwork for future studies in dogs aimed at understanding the underlying pathophysiology of this disease.

Because the mutation identified here arose de novo in this particular litter, it is unlikely that the same mutation would be present in other dogs with osteochondromatosis. Therefore, a genetic test based upon this specific mutation would be unlikely to provide any benefit to diagnosis in other dogs. However, given the genetic epidemiology in humans, which has documented >600 known mutations in EXT1 and EXT2 associated with the disease, future cases of osteochondromatosis in dogs could be confirmed by sequencing both genes in their entirety in affected animals. This type of sequencing-based test could be helpful to breeders, especially in identifying cases that appear to be heritable across generations rather than de novo events.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Authors declare no IACUC or other approval was needed.

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