

A neonatal encephalopathy with seizures in standard poodle dogs with a missense mutation in the canine ortholog of *ATF2*

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Abstract Neonatal encephalopathy with seizures (NEWS) is a previously undescribed autosomal recessive disease of standard poodle puppies. Affected puppies are small

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and weak at birth. Many die in their first week of life. Those surviving past 1 week develop ataxia, a whole-body tremor, and, by 4 to 6 weeks of age, severe generalized clonic–tonic seizures. None have survived to 7 weeks of age. Cerebella from affected puppies were reduced in size and often contained dysplastic foci consisting of clusters of intermixed granule and Purkinje neurons. We used deoxyribonucleic acid samples from related standard poodles to map the NEWS locus to a 2.87-Mb segment of CFA36, which contains the canine ortholog of *ATF2*. This gene encodes activating transcription factor 2 (ATF-2), which participates in the cellular responses to a wide variety of stimuli. We amplified and sequenced all coding regions of canine *ATF2* from a NEWS-affected puppy and identified a T>G transversion that predicts a methionine-to-arginine missense mutation at amino acid position 51. Methionine-51 lies within a hydrophobic docking site for mitogen-activated protein kinases that activate ATF-2 so the arginine substitution is likely to interfere with ATF-2 activation. All 20 NEWS-affected puppies in the standard poodle family were homozygous for the mutant G allele. The 58 clinically normal family members were either G/T heterozygotes or homozygous for the ancestral T allele. There are no previous reports of spontaneous *ATF2* mutations in people or animals; however, *atf2*-knockout mice have cerebellar lesions that are similar to those in puppies with NEWS.

Keywords *ATF2* · Activating transcription factor 2 · Neonatal encephalopathy · Seizures · Dogs

Introduction

Human neonatal encephalopathy in full-term or near-term infants is clinically defined as a constellation of signs that include abnormal consciousness, tone and reflexes, feeding, respiration, or seizures that can result from a myriad of etiologies [1]. Seizures associated with neonatal encephalopathies are often intractable to conventional antiepileptic drugs and often carry a grave prognosis [2]. Dogs that fail to thrive and die within hours or days of birth because of an encephalopathy or a wide variety of other etiologies are often referred to as fading puppies. The condition is common [3], and most experienced dog breeders expect to produce occasional fading puppies. Nonetheless, between 1997 and 2005, several breeders of standard poodles encountered an unusually high incidence of fading puppies. These puppies exhibited specific neurologic signs, which included seizures if they survived beyond 4 weeks of age; moreover, necropsied puppies had a characteristic encephalopathy. Hence, we refer to this previously undescribed canine disease as neonatal encephalopathy with seizures (NEWS).

We here describe the clinical signs of NEWS, the mapping of the NEWS locus, and the identification of a missense mutation in the canine ortholog of *ATF2* in deoxyribonucleic acid (DNA) from NEWS-affected puppies. *ATF2* encodes activating transcription factor 2 (ATF-2), a widely expressed member of the basic region-leucine zipper (bZIP) family of transcription factors [4]. This transcription factor functions as a homodimer or as a heterodimer with other bZIP family members including c-Jun, JunB, JunD, c-Fos, Fos-B, FRA2, ATF-3 and C/EBP γ [5]. ATF-2 is involved in the regulation of a variety of cellular processes including proliferation [6], differentiation [7, 8], adaptation to a variety of stressors [9, 10], oncogenic transformation [11], and apoptosis [12]. ATF-2 is required for the normal development and function of the central nervous (CNS) [13], respiratory [14], immune [15], reproductive [16], renal [17], and skeletal systems [18].

Materials and methods

Samples and clinical evaluations

Ethylenediamine tetraacetic acid blood and/or frozen tissues were collected from 78 members of a large standard poodle family with the informed consent of the dogs' owners. DNA was isolated from blood leukocytes or tissue (dew claws, spleen, or tongue) as previously described [19]. In addition, the owners provided us with information about the health of all 78 of the dogs. We received video tapes showing five affected puppies and formalin-fixed brain

tissue collected at postmortem from 14 affected puppies. One affected female puppy was presented to the University of Missouri Veterinary Teaching Hospital at 3 weeks of age and observed until she was euthanized because of intractable seizures in her sixth week of age. When the puppy was 4 weeks old, we recorded an interictal electroencephalogram (EEG) while she was conscious. We used a simple five-lead montage with each frontal and occipital area referenced to the vertex (left frontal, left occipital, right frontal, right occipital, vertex) [20]. The EEG was recorded on a Cadwell Sierra Central Lab with high- and low-pass filters set at 70 and 1.0 Hz, respectively.

Linkage analysis

We used a highly multiplexed panel of 304 autosomal microsatellite markers for linkage analysis as described by Clark et al. [21]. We generated additional CFA36 microsatellite markers for fine mapping by examining targeted regions of the canine genome sequence (http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=9615) and searching for single-copy segments containing dinucleotide repeats with copy numbers of at least 18. Primers were designed with Oligo[®]6 software. The primer sequences and chromosomal locations for all CFA36 markers used in this study are in Table 1. Microsatellite genotypes were examined for misinheritance and incorrect scoring using GENOPROB [22, 23], and genotypes with low probability ($p_{\text{Gmx}} < 0.95$) were excluded from further analysis. Logarithm of the odds ratio (LOD) scores were determined by multipoint linkage analysis of the NEWS locus on CFA36 using the Mendel software [24] assuming complete penetrance and that 1 cM is equivalent to 1 Mb uniformly across the entire chromosome.

Sequencing and pyrosequencing canine *ATF2*

The nucleotide sequence for the region from 22.05 to 22.12 Mb on CFA36, which contains *LOC478806*, the canine ortholog of *ATF2*, was downloaded from the canine genome sequence assembly (build 2.1). From this sequence, we identified the 12 canine *ATF2* exons using Model Maker of the National Center for Biotechnology Information, and we used Oligo[®]6 to design polymerase chain reaction (PCR) primers flanking the coding regions of each exon (Table 2). All exons were amplified and sequenced as previously described [19]. The predicted consequence of a transversion identified in exon 3 was estimated using the position-specific independent counts (PSIC) calculated on the PolyPhen server (<http://genetics.bwh.harvard.edu/pph/>) [25].

We used a pyrosequencing assay to genotype at the transversion in exon 3 of canine *ATF2*. The assay was

Table 1 CFA36 microsatellite markers

Marker	CFA36 position (K)	Forward primer sequence/reverse primer sequence 5'–3'	Amplicon size (bp)
<i>UMC0288</i>	4,369	TAAAACAAATGACCCTAATGTCTTAGAA/ TCCTGCTGAGACTACAAGAGTAGGAA	218
<i>FH2611</i>	9,234	GAAGCCTATGAGCCAGATCA/TGTTAGATGATGCCTTCCTTCT	208
<i>REN179H15</i>	16,694	AGCCATTTGCCAACCTACAC/AACACACACCAGTTTGGCA	314
<i>UMC0335</i>	18,999	AATCATAAATGACGTTGCCTAATAAC/CCCCATGTAGCCACAATCTGC	250
<i>UMC0296</i>	19,286	CCACCTCCACTCCAATCACAAC/GCAATGGTATGAAACATCAAGGGTAGT	185
<i>UMC0297</i>	20,411	CAAACGAAAGCACATCTCCGTAAG/GCCAGTCCACTGACCTGCTACAT	255
<i>UMC0337</i>	20,832	AGGAAATAAGGTGCCATGCATCT/TTGGGCTGCTCTCTTTATGTGAC	156
<i>UMC0338</i>	21,451	TGGCATAGGGAATTTCAACTTATTCA/CACCGGGCTCCTTGTAG	198
<i>UMC0298</i>	22,037	ATGTCGTCGTTTCGGCCATC/CCGTCCTCCCACTTGCCTC	276
<i>UMC0340</i>	22,670	AAAATTAATGTATTCACAGACAATGGCT/CCACACCACCCCTTCTGATATGT	199
<i>UMC0289</i>	23,284	AGCACCTGGAGGCATAATAACAT/TGATTAAATGGCATGGGTATTCA	315
<i>FH3865</i>	23,292	CTCCCCACAGGAAGTCTG/CTACCTTCCCAAGCTAAGG	401
<i>UMC0299</i>	23,821	CAGCAGCAACGCCACTCAATC/AGTTCAAGAAGGTGCGGTAGCTC	310
<i>REN252E18</i>	25,428	CAGCATTTCTCACTTTCCC/GGGGAGATTGTGTATCGGAA	259
<i>UMC0290</i>	31,339	ATATCAGGCAAGGCAGATACCAG/GGGAGATGGGTGAAATTGGTG	249
<i>DTR36.3</i>	31,474	TTCTAACCAGCTACCAGAG/TGTTGATATTTGATGTTGCC	211

performed with a PSQ 96 Pyrosequencer as directed in the PSQ Sample Preparation Guidelines (Biotage, Charlottesville, VA). The PCR primers were 5'-biotinyl-AGGAT CATTGGCTGTCCATA-3' with 5'-TATACTAACCAGC CACAATGACACT-3', and 5'-CTGGACCAATTT CAGTGTC-3' was used for the sequencing primer.

Results

Clinical presentation

We collected DNA and clinical histories from 78 members of a family of standard poodles (Fig. 1). Twenty family members died as puppies from NEWS. Affected puppies

were smaller at birth than their littermates. They nursed poorly and often required supplemental nutrition during their first 2 days of life. Affected puppies nursed adequately after the first few days but failed to develop normally. Puppies that survived to 3 weeks of age had a marked whole-body tremor, weakness, and ataxia. Some were able to walk a few steps but had a wide-based stance with increased extensor tone and frequently fell. Axial muscle weakness led to ventroflexion of the neck in some pups. While the weakness may have contributed, the tremors and ataxia were more characteristic of cerebellar dysfunction. Affected puppies did not interact with their littermates or mother, and their responses to external sensory stimuli were sluggish and incomplete. Between 4 and 6 weeks of age, they developed severe generalized clonic-tonic seizures (Supplementary Video 1). In the one

Table 2 Amplification of canine *ATF2* exons

Exon	Forward primer sequence/reverse primer sequence	Amplicon size (bp)
1	AGGTTTGGGGTTGACAGTGTTA/TTCCAAATACCGCATAACATA	210
2	TCCTAATGTTTTATTATAGGCAGTACA/TTTAGTGACAACATAAACTATCATCAGG	166
3	TGCTTATTCTTGAGTAATGGGTT/GTGCACATTCCAGGTGAAATATGTATA	354
4	AGCTATATTTGAGAGTCTGCATATT/AAATGCCAGTCAGTATATCTAAAGTAAGA	277
5	AAGAAAGCCTCAGAAGACGACAT/CACAAATAAGGCTATTACAACCTAA	343
6	ATATTCTGTTCACTCTAACGTCATGGA/ACTAGCCCAAATCATCACTTAC	275
7	CATATTTTTAGAGATGACTAATAAGGATT/CAGTTTGGTATGAAGAAAAGATTTAGTCT	224
8	ATAGCCATTGTGCAGATATAGGAC/GGCTTTAATGGAAAACTAATTCA	226
9	CTGGGAGTTGAATTTGAATGGA/TAAAGGATGATAACAGAGCATGAATGA	259
10	TGTGCCTGTGTTCTCACTGGATACAAA/ATGGGAAAAACAGGGAAGAATAAGT	336
11	GAAATGTTTAGTCTGATGCTGTTAAGGT/CGCTCTGGCAGTATTTCACTCAT	215
12	CAAACCTGTTGACACATTCCTAGAGATGTT/GATTTCCCTTGAAGTCACTAATGAGT	348

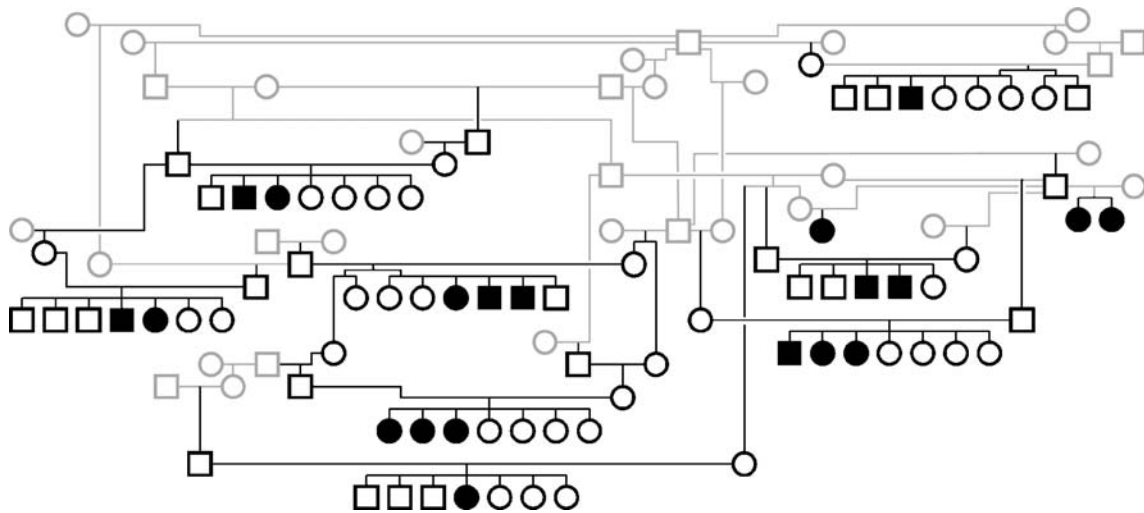


Fig. 1 Pedigree shows relationships among the standard poodle family members used for mapping. DNA samples were available from dogs represented by *black figures* but not from dogs represented by

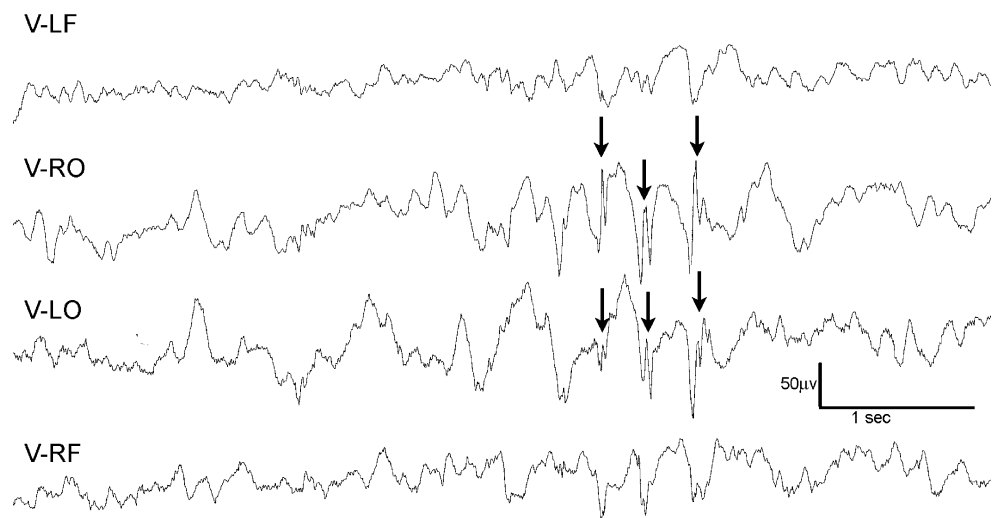
gray figures. Puppies affected with NEWS are represented by *solid figures*; asymptomatic canine family members are represented by *open figures*

recorded puppy, the interictal EEG demonstrated spikes and slow waves indicative of seizure activity (Fig. 2). In this pup, the seizures were treated with oral phenobarbital. Initially, therapy controlled the seizures, but she became refractory to treatment within 2 weeks. Serum concentrations of glucose, electrolytes, and ammonia were within normal limits in all four tested puppies. As the disease progressed, affected puppies became laterally recumbent with extensor rigidity and opisthotonus. All affected puppies died or were euthanized because of unrelenting seizures and/or declining neurologic status before they reached 7 weeks of age. Cerebella from affected puppies were reduced in size and often contained dysplastic foci consisting of clusters of intermixed granule and Purkinje neurons (Fig. 3).

Whole-genome mapping

NEWS segregated as an autosomal recessive trait in the standard poodle family (Fig. 1). A whole-genome scan for the NEWS locus was performed by genotyping all standard poodle family members with a panel of 304 canine microsatellite markers [21]. Multipoint linkage analysis placed the disease locus on CFA36 with LOD scores exceeding 7.0 between markers *REN179H15* at 16.69 Mb and *REN252E18* at 25.43 Mb (Fig. 4). Eleven additional CFA36 microsatellite markers (Table 1) were used for fine mapping the NEWS locus. Recombinant CFA36 chromosomes established *UMC0297* and *REN252E18* as the respective centromeric and telomeric boundaries for the NEWS locus (Fig. 5).

Fig. 2 An EEG from a 4-week-old puppy with NEWS showing spikes (*arrows*) and slow waves most prominent in the occipital leads



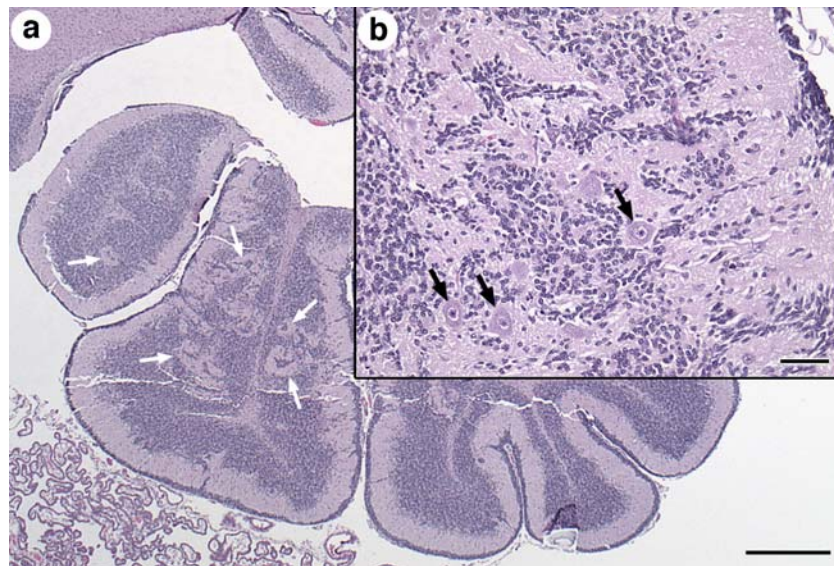


Fig. 3 Low- and high-powered photomicrographs of hematoxylin-and-eosin-stained sections from the cerebellum of a 6-week-old standard poodle with NEWS. **a** Arrows point to dysplastic foci in which Purkinje neurons and surrounding regions of the molecular

layer are embedded in the internal granular layer. Scale bar represents 500 μ m. **b** Arrows point to Purkinje neurons within a dysplastic focus. Scale bar represents 50 μ m

We next examined the genotypes of all 20 affected standard poodles at the seven marker loci between *UMC0297* and *REN252E18* and identified two haplotypes harboring the disease allele (Table 3). Fourteen of the NEWS-affected standard poodles were homozygous for haplotype 1, and the remaining six were heterozygous for the two haplotypes. The existence of two haplotypes harboring the NEWS allele is almost certainly attributable to a meiotic recombination that occurred in an ancestor of the poodles in this family. The putative recombination event

occurred between a NEWS haplotype and a wild-type haplotype in the chromosomal segment flanked by *UMC0340* and *UMC0289*. All 20 affected standard poodles were homozygous for the four markers between *UMC0297* and *UMC0289*; consequently, we focused our efforts to identify the NEWS mutation on the genes that occupy this 2.87-Mb segment of CFA36.

A missense mutation in canine *ATF2*

The targeted 2.87-Mb CFA36 segment contains part, or all, of 26 canine orthologs of HSA2 genes, at least three of which (*CHN1*, *ATF2* and *HOXD1*) are potentially involved in the development of the CNS [26]. We started with *LOC478806*, the canine ortholog of human *ATF2*, and sequenced all 12 exons with template DNA from an affected puppy. This revealed a c.152T>G transversion in exon 3 (GenBank accession number EF188808) that predicts a methionine-to-arginine missense mutation at amino acid position 51. Analysis of the methionine-to-arginine substitution on the PolyPhen server produced a delta-PSIC score of 3.120. Delta-PSIC scores greater than 2.0 are interpreted to indicate that the amino acid substitutions will be “probably damaging” to protein function [25].

We used a pyrosequencing assay to genotype all 78 standard poodle family members at this transversion. All 20 affected puppies in the family were homozygous for the mutant G allele. All 16 of the available DNA samples from clinically normal obligate carriers were G/T heterozygotes, while the remaining 42 clinically normal family members were either G/T heterozygotes ($n=32$) or were homozygous for the ancestral T allele ($n=10$). The G allele was absent

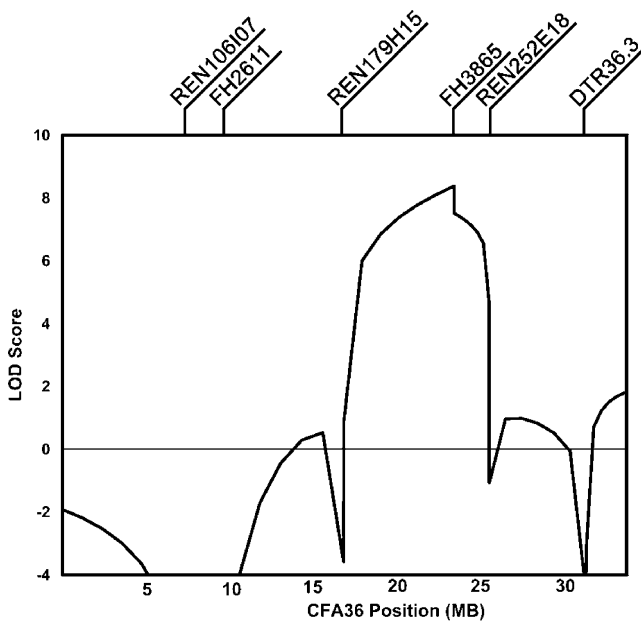
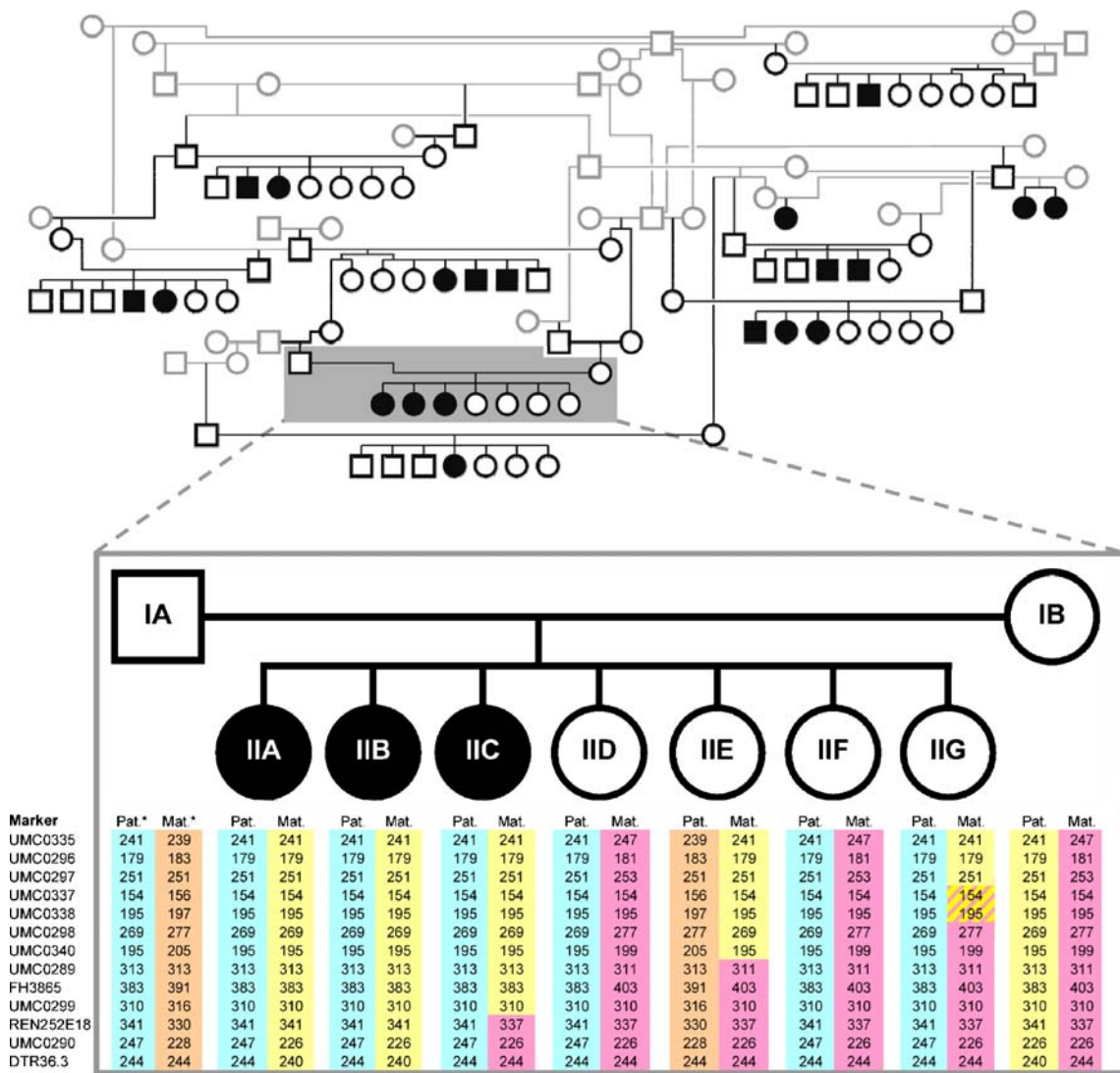


Fig. 4 Multipoint linkage analysis assigns the NEWS locus to CFA36



*Pat. = paternal chromosome; Mat. = maternal chromosome

Fig. 5 Fine mapping of the NEWS locus. Three CFA36 recombinations were detected in the subfamily that is shaded in the pedigree (top). NEWS-affected puppy IIC inherited a paternal chromosome harboring the disease allele and a maternal chromosome that was recombinant between UMC0299 and REN252E18, which localizes the NEWS locus centromeric to REN252E18. The recombinant maternally inherited chromosome in puppy IIE is not informative for the location

of the NEWS locus because the paternally inherited chromosome harbors the wild type allele and the puppy has the dominant normal phenotype. Phenotypically normal puppy IIG inherited a paternal chromosome harboring the disease allele and a maternal chromosome that was recombinant between UMC0297 and UMC0298, which establishes the NEWS locus as being telomeric to UMC0297

from the DNA of 118 unrelated dogs representing 93 other dog breeds.

Since discovering the association of the *ATF2* mutation with NEWS, we have provided a genotyping service to breeders and owners of standard poodles. As of September

2007, we have genotyped 1,038 standard poodles and have found 638 to be homozygous for the ancestral T allele, 371 to be heterozygous T/G carriers, and 29 to be affected G/G homozygotes. All 29 of the G/G homozygotes exhibited NEWS symptoms, and none survived into their seventh

Table 3 Haplotypes in NEWS-affected dogs

MARKER	UMC0337	UMC0338	UMC0298	UMC0340	UMC0289	FH3865	UMC0299
Position (Mb)	20.832	21.451	22.037	22.670	23.284	23.292	23.821
Haplotype 1 (bp)	154	195	269	195	313	383	310
Haplotype 2 (bp)	154	195	269	195	309	383	305

week of age. In contrast, all of the T/G heterozygotes and all but two of the T/T homozygotes survived past their first few months of life, and neither of the mortalities exhibited neurologic signs typical of puppies with NEWS. One puppy died from an accidental traumatic injury and the other from unknown causes. Thus, NEWS appears to be a fully penetrant autosomal recessive disease.

Discussion

We collected DNA from an extended family of standard poodles and mapped the NEWS locus to a 2.87-Mb segment of CFA36 by linkage analysis and homozygosity mapping. This chromosomal region harbors the canine ortholog of *ATF2*. Within this gene, we identified a T to G transversion that predicts the substitution of an arginine for a methionine at amino acid position 51. All 29 of the standard poodles in our study that exhibited NEWS symptoms were homozygous to the argininy allele, whereas none of the more than 1,000 standard poodles surviving past 7 weeks of age were homozygous for the argininy allele.

The perfect concordance between the NEWS phenotype and homozygosity for the argininy allele could be explained if the argininy allele were in tight linkage disequilibrium with an unidentified mutation that causes NEWS. Nonetheless, there are several reasons to suspect that the c.152T>G mutation is itself the direct cause of NEWS. For one thing, the NEWS phenotype has not been recognized in breeds other than the standard poodle. The argininy allele occurred frequently in DNA samples from standard poodles; however, it was not detected in more than 200 chromosomes in randomly selected representatives of other breeds. In addition, a BLASTP (protein-protein BLAST) search revealed that methionine 51 and the flanking ten amino acid sequences on either side of M-51 are perfectly conserved in human ATF-2 and in the ATF-2 sequences from eight other mammalian species.

Furthermore, two different strains of *atf2*-knockout mice have been characterized [13, 14], and similarities between these mice and affected puppies suggest that NEWS is caused by an ATF-2 deficiency. Nullizygous *atf2*-knockout mice lacking any detectable *atf2* transcripts died at birth from respiratory failure [14]; however, homozygous knockout mice from an earlier strain were able to breathe [13], possibly because they benefited from a partially functional *atf2* splice variant [14]. About half of these homozygous mice died when less than 1 month old. The other half had a normal lifespan, but they were ataxic, abnormally small, and had a marked whole-body tremor. Postmortem abnormalities of the *atf2*-nullizygous mice included disorganized epiphyseal growth plates, atrophic vestibular sense organs, and an intermingling of displaced Purkinje neurons and granular layer neurons of the cerebellum [13].

The standard poodles with NEWS resembled the *atf2*-knockout mice in that they were smaller than their littermates at birth and would die within the first 48 h after birth if not given supplemental nutrition and nursing care. Also similar to the *atf2*-mice, affected puppies were ataxic and had a marked whole-body tremor. Although seizures were not reported in the *atf2*-knockout mice, the NEWS puppies that survived to 5 weeks of age developed severe, generalized seizures with spike and wave discharge on EEG. The most obvious postmortem abnormalities observed in the NEWS-affected puppies were the decreased size and disrupted layering of their cerebella, which was also reported for the *atf2*-knockout mice [13] but is seldom observed in other encephalopathies. Abnormalities elsewhere in the CNS of affected dogs are expected because cerebellar pathology alone is unlikely to account for the seizures and mental changes of these puppies. An in-depth examination of all regions of the CNS and other tissues from NEWS-affected puppies is underway, and the findings will be reported elsewhere.

The transcription factor and acetyltransferase activities of ATF-2 are promoted by phosphorylations at Thr-69 and Thr-71 [27, 28]. These phosphorylations are catalyzed by a variety of mitogen-activated protein kinases (MAPKs) including the c-Jun N-terminal kinases, extracellular signal-regulated protein kinase 2, and the p38 MAPKs [29–31]. Homozygous *atf2*-knockin mice, in which the threonines corresponding to Thr-69 and Thr-71 have been replaced with alanines, die at birth [32]. These mice resemble the *atf2*-knockout mice that died from respiratory failure [14], underscoring the *in vivo* importance of ATF-2 activation. Codon-51 of canine *ATF2*, which contains the NEWS-associated transversion, is located immediately adjacent to the alpha-helix that forms the backbone of the zinc finger in the transactivation domain of ATF-2 [33]. Met-51 contributes to a hydrophobic region on the protein surface that forms part of a MAPK docking site [34]. This docking site facilitates the specific and efficient phosphorylation of Thr-69 and Thr-71, thereby activating the transcription factor. Homologous MAPK docking sites occur in a variety of other proteins, all of which have hydrophobic amino acids (leucine, valine, isoleucine, or methionine) at the position corresponding to Met-51 in ATF-2 [34, 35]. Thus, the substitution of arginine at this position is likely to disrupt the docking site and interfere with *ATF2* activation in affected standard poodles.

The chromosomal location of the NEWS locus and the similarities between the symptoms and the cerebellar lesions in NEWS-affected puppies and *atf2*-knockout mice suggest that NEWS is caused by an ATF-2 deficiency. The absolute amino acid sequence conservation at and flanking codon 51 among mammalian ATF-2s and the probability that the substitution of a basic argininy residue at this position will

disrupt the hydrophilic MAPK docking site and interfere with ATF-2 activation suggest that the c.152T>G transversion is the direct cause of NEWS in standard poodles. Thus, it is not surprising that this nonsynonymous SNP received a high PolyPhen score, which predicts that the transversion is “probably damaging” to *ATF2* function [25].

The discovery that an allele in canine *ATF2* is associated with NEWS is of interest to standard poodle breeders who want to identify the asymptomatic carriers in their breeding stock. Standard poodle breeders can avoid future cases of NEWS by ensuring that carriers are only bred to homozygous normal mates. Nonetheless, it would be prudent to monitor the health of carriers because an unusually high incidence of breast cancer has been observed among the heterozygous breeding stock in colonies of *atf2*-knockout mice [36, 37].

Neonatal encephalopathy is a significant contributor to mortality and morbidity in full-term infants with a prevalence of 3.2–4.4 per 1,000 live term births [38]. Although neonatal encephalopathy is often associated with intrapartum hypoxia, recent reports indicate that over two thirds of neonatal encephalopathy cases could be attributed to a variety of antepartum factors, including genetic risk, and not to birth asphyxia [1, 38–40]. There are no previous reports of human or domestic animal diseases caused by *ATF2* mutations; however, the spontaneous occurrence of NEWS in standard poodles suggests that *ATF2* mutations may be responsible for instances of encephalopathy in infants and in neonates of other species. Considering the numerous key functions ascribed to ATF-2 in various cell types, it is remarkable that NEWS-affected puppies have survived beyond birth and into their sixth week of life. The severity of the canine *ATF2* mutation may be mitigated because the mutant Arg-51 ATF-2 retains partial biological activity. Furthermore, redundant or compensatory biological pathways may substitute for pathways that depend on normal ATF-2 function.

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