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Autosomal Dominant Canine Malignant Hyperthermia Is Caused by a Mutation in the Gene Encoding the Skeletal Muscle Calcium Release Channel (RYR1)

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Background: **Malignant hyperthermia (MH) is an inherited disorder of skeletal muscle characterized by hypercarbia, rhabdomyolysis, generalized skeletal muscle contracture, cardiac dysrhythmia, and renal failure, that develops on exposure to succinylcholine or volatile anesthetic agents. All swine and up to 50% of human MH events are thought to be associated with mutations in the calcium release channel of the sarcoplasmic reticulum, also known as the ryanodine receptor (*RYR1*). Events resembling MH have been reported in other species, but none have undergone genetic investigation to date.**

Methods: **To determine the molecular basis of canine MH, a breeding colony was established with a male, mixed-breed, MH-susceptible (MHS) dog that survived an *in vivo* halothane–succinylcholine challenge. He was mated to three unaffected females to produce four litters and back-crossed to an affected daughter to produce one litter. One of his MHS sons was mated to an unaffected female to produce an additional litter. Forty-seven dogs were phenotyped with an *in vitro* contracture test and diagnosed as MHS or MH normal based on the North American *in vitro* contracture test protocol. Nine microsatellite markers in the vicinity of *RYR1* on canine chromosome 1 (CFA01) were tested for linkage to the MHS phenotype. Mutational analysis in two MHS and two MH-normal dogs was performed with direct sequencing of polymerase chain reaction products and of cloned fragments that represent frequently mutated human *RYR1* regions. A restriction fragment length polymorphism was chosen to detect the candidate mutation in the pedigree at large.**

Results: **Pedigree inspection revealed that MHS in this colony is transmitted as an autosomal dominant trait. FH2294, the marker closest to *RYR1*, is linked to MHS at a $\theta = 0.03$ with a LOD score of 9.24. A T1640C mutation gives rise to an alanine for valine substitution of amino acid 547 in the RYR1 protein, generating a maximum LOD score of 12.29 at $\theta = 0.00$. All dogs diagnosed as MHS by *in vitro* contracture test were heterozygous for the mutation, and all MH-normal dogs were homozygous for the T1640 allele.**

Conclusions: **These results indicate that autosomal dominant canine MH is caused by a mutation in the gene encoding the skeletal muscle calcium release channel and that the MHS trait in this pedigree of mixed-breed dogs is in perfect cosegregation with the *RYR1* V547A mutation.**

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MALIGNANT hyperthermia (MH), a pharmacogenetic disorder of skeletal muscle elicited by exposure to volatile anesthetics and depolarizing muscle relaxants, is well recognized in humans and pigs, although a number of definite episodes have also been reported in dogs.[1–7] When given these agents, MH-susceptible (MHS) dogs show tachycardia, hyperthermia, elevated carbon dioxide production, and death if the anesthetic is not discontinued. Specific interventions, including use of the calcium release channel antagonist dantrolene,[8,9] are efficacious in reversing signs of the canine syndrome. In most reports of MH in dogs, metabolic acidosis is moderate and muscle rigidity is minimal, in contrast to the severity of both in the swine or human condition. Many additional accounts of episodes resembling MH in dogs within the perioperative interval,[10–13] during exertion,[14–17] or other chemical exposures[18] have been published. Monitoring and laboratory investigations in these descriptions are scant, and uncertainties persist regarding the incidence of canine MH, as well as its relation to other disorders in the dog, and to MH in other species.

To resolve these issues, Nelson[19] assembled a pedigree for *in vivo* halothane–succinylcholine challenge and *in vitro* contracture testing (IVCT), beginning with a Doberman–German Shepherd–Collie mixed-breed progenitor from a colony maintained by the late Dr. Barry Reynolds at the University of Saskatchewan. Members of the pedigree produced by the mating of this dog to an MH-normal (MHN) Labrador Retriever dam were challenged under well-controlled anesthetic dosing regimens. Contemporary monitors and serial laboratory assays were used to discriminate features shared with human and swine MH from those that were distinct, and

to establish the predictive value of the IVCT in a third species. Subsequently, a male subject in these experiments became the propositus of a second colony bred for molecular genetic diagnosis. His mates and all viable descendants were IVCT phenotyped to ascertain MH status, and *in vivo* halothane–succinylcholine challenge tests were simultaneously conducted in all dogs from four of the six sibships. Because swine and many human probands are predisposed to MHS by mutations in the calcium release channel gene (*RYR1*),[20] cosegregation was sought between inheritance of the canine MHS trait and *RYR1* as a candidate locus. Initial experiments ruled out the *RYR1* mutation shared between all MHS swine (R614C) and 2–7% of human MHS (R615C) pedigrees.[21] The canine pedigree was then genotyped with polymorphic DNA markers linked to the *RYR1* locus on canine chromosome 1 (CFA01).

Materials and Methods

Malignant Hyperthermia Dog Breeding Colony

All investigations were conducted with the approval of the Institutional Animal Care and Use Committees of the University of Wisconsin (Madison, WI), Wake Forest University (Winston-Salem, NC), and the University of Texas (Houston, TX). To assemble the breeding colony, a MHS Doberman–German Shepherd–Collie–Labrador Retriever mixed-breed sire was selected from a previously reported kindred[19] and was out-crossed to three unrelated, mixed-breed MHN females to produce four litters, and back-crossed to one MHS daughter to produce one litter. An MHS male offspring (#6-5450) was also mated to an unrelated mixed-breed MHN female. These pairings produced 34 F1 and 13 F2 siblings. Including all parents, a total of 52 dogs were available for genotyping, of which 47 were phenotyped by IVCT.

In Vivo *Halothane–Succinylcholine Challenge Testing*

Dogs were anesthetized with 25 mg/kg intravenous pentobarbital, the trachea was intubated, and the lungs were mechanically ventilated with 30% O_2 and 70% N_2O at volumes sufficient to maintain end-tidal carbon dioxide at 35–40 mmHg partial pressure. After removal of gracilis muscle bundles for contracture testing, the femoral artery was cannulated for sample collection and arterial pressure transduction. Halothane (2% inspired in oxygen) and intravenous succinylcholine (0.3 mg/kg body weight) were administered as previously described,[19] and intravenous dantrolene (0.2 mg/kg body weight every 3 min to a total dose of 3.0 mg/kg) was administered when life-threatening tachydysrhythmias, acidosis, or hyperthermia developed. Thirty-one dogs were tested with *in vivo* challenge anesthetics, including all parents, and all dogs in sibships No. 3–6. Sixteen pups in sibships No. 7 and 9 did not undergo *in vivo* challenge testing. By the time these dogs were large enough for IVCT assays, the correlation between MHS and IVCT was sufficiently established to preclude the additional costs and euthanasia of further *in vivo* challenges. Five pups from the back-cross litter of eight were either stillborn or died shortly after birth, precluding ascertainment of the MH phenotype by any method.

In Vitro *Contracture Testing*

Specimens of gracilis muscle underwent biopsy immediately preceding the *in vivo* test using protocols approved by North American MH Group for human MH diagnosis.[19,22] Fasicle viability for contracture testing was assessed before exposure to caffeine and halothane by electrically evoked contracture tensions. For all fasicles from all MHS and MHN dogs, the contracture tension averaged 8.24 ± 0.24 g (mean ± SD). Three tests were performed on muscle fasicles that underwent biopsy: increment in contracture tension (grams) in the presence of 3% halothane, caffeine specific concentration (CSC; millimolars) required to generate a 1 g isometric contracture, and the halothane CSC (millimolars) reflecting a measure identical to the CSC but performed in the presence of 1% halothane. Bundles from all dogs were tested in triplicate for the 3% halothane test and were triply replicated in the majority for the CSC and halothane CSC as permitted by the availability of viable fasicles.

Genomic DNA Isolation

Genomic DNA of each canine subject was extracted either from EDTA-preserved whole blood in dogs undergoing IVCT or from frozen muscle in the nonviable back-cross animals, using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN).

Selection of DNA Markers

Canine *RYR1* was located on chromosome 1 (CFA01) by radiation hybrid mapping.[23]†† Polymorphic microsatellite markers from CFA01 were selected from an integrated physical–genetic linkage map with appended polymerase chain reaction (PCR) primers specific for each marker locus.[24] CFA01 marker order in the region is REN143K19-RYR1-FH2294-FH2326-C01.164-FH2309-C01.251-C01.246-FH2313-FH2016, spanning 90.7 centiMorgans and 639.6 centiRays.

Genotyping

Polymerase chain reactions containing 12.5 ng DNA, PCR Buffer (Qiagen, Inc., Valencia, CA), 1.5 mM $MgCl_2$, 5 pmol each of specific forward and reverse primers, 25 μM each of dCTP, dGTP, and dTTP, 6.25 μM dATP,

†† Additional information can be found at the following Web site: Hitte C: The Canine Radiation Hybrid Project. Available at: www-recomgen.univ-rennes1.fr/Dogs/doggy-home.html. Accessed July 15, 2001

Table 1. List of Primers for the Overlapping PCR Products Used for Sequencing

PCR Product	Primer Sequence		Product Size (bp)
	Forward	Reverse	
RYR1-1	CCT CGA CAT CAT GGG TGA CG human bases 96–115	TAG TAG ACA AGT CTG CGC TG human bases 842–823	746
RYR1-2	TGT GGA ACA TGA ACC CCA TCT human bases 698–718	TTG CTC TGC TTC TCC TCG TG human bases 1502–1483	804
RYR1-3	TTC ATC AAG GGC CTG GAC AG human bases 1342–1361	TCC ACC ATC ACC TCA AAG TAC human bases 2108–2088	759
RYR1-4	GGA GTA TTT CTG TGA CCA AG human bases 5931–5950	GGG TGT TGG TAG AAG ACT TT human bases 6686–6667	755
RYR1-5	CTG GTG ATC GTG CAG ATG GG human bases 6595–6614	GGT CGA TCA AGG CAG CAT AG human bases 7396–7377	801
RYR1-6	TAC CTG GAC TTC CTG CGC TT human bases 7093–7112	AAC TCG TTA AGG ATC CCG AC human bases 8009–7990	916
RYR1-1416	TGG TCC TGA ACT GTA TTG AC	CGT GCT TGT CCA GGA GGG	487

PCR = polymerase chain reaction; bp = base pairs; RYR1 = ryanodine receptor.

4.625 kBq [α-^{33}P] dATP, and 0.3 units HotStarTaq polymerase (Qiagen, Inc.) in a final volume of 15 μl were performed in 96-well plates with initial denaturation at 94°C for 15 min; 30 cycles of 92°C for 30 s, 56°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 5 min using a PTC-100 thermocycler (MJ Research, Inc., Incline Village, NV). The reaction products were observed by electrophoresis through 5% acrylamide denaturing gels on BioRad SequiGen GT 38 × 50-cm plate sequencing gel units (BioRad Laboratories, Hercules, CA), and alleles were sized by comparison to an M13 sequence ladder after autoradiography.

RYR1 *cDNA Synthesis*

[1]mRNA was isolated from 200 mg of skeletal muscle harvested from two MHN (#3-5346 and #9-5504) and 2 MHS (#3-5348 and #9-5507) dogs using the Micro Fast Track 2.0 mRNA isolation kit (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized using 1.0 μl of mRNA, either 75 ng random hexamers or 1.5 μM RYR1-6.Reverse primer (table 1), 2.5 mM $MgCl_2$, 0.5 mM dNTPs, 10 mM DTT, and 100 units of BRL Superscript II Reverse Transcriptase (Life Technologies, Rockville, MD).

RYR1 *cDNA Sequencing*

Polymerase Chain Reaction Templates. Two regions of the *RYR1* cDNA were selected for sequencing based on the frequency of previously identified mutant sites in humans and swine.[20]‡‡ Region I, containing bases 22–1982, was sequenced by overlapping PCR products RYR1-1, RYR1-2, and RYR1-3 (table 1). Region II, containing bases 5946–7927, was sequenced by overlapping PCR products RYR1-4, RYR1-5, and RYR1-6 b (table 1). PCR primers designed to reflect consensus pig, human, and rabbit sequences are shown in table 1. PCR amplification of *RYR1* cDNA templates used 0.8 μg cDNA in 1.0 μl, 200 nM of each primer, 5.0 μl of buffer (Qiagen, Inc.), with 1.5 mM $MgCl_2$, 200 μM dNTPs, and 0.5 units of HotStarTaq in a 50-μl PCR reaction. The cycling conditions were an initial denaturation at 94°C for 15 min; 30 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 30 s; and a final extension at 72°C for 5 min. PCR products were sequenced in the forward and reverse directions using an ABI 377 by the Advanced Genetic Analysis Center of the University of Minnesota.

Cloned Templates. Because disproportionate representation by PCR amplification may conceal heterozygous alleles on direct sequencing, RYR1-3 PCR products from the MHS dog No. 3-5348 were also cloned. PCR products were ligated into Invitrogen vector pCR-TOPO, transformed into *Escherichia coli* competent cells (Invitrogen, Carlsbad, CA), and plated on Luria Bertani–ampicillin plates. Twenty-two colonies were picked for PCR analysis.

Sequencing RYR1: Genomic DNA

Polymerase chain reaction primers RYR1-14.Forward and RYR1-16.Reverse (table 1) were designed to amplify the fragment between exons 14 and 16 (RYR1-1416) from genomic DNA templates based on the canine sequence of PCR product RYR1-3, and the known intron–exon boundaries of human *RYR1*. The reaction consisted of 25 ng genomic DNA, 40 μM dNTPs, 1.5 μl PCR buffer with 1.5 mm $MgCl_2$ (Qiagen, Inc.), 0.3 units of HotStarTaq (Qiagen, Inc.), and 0.67 μM of each primer in each 15-μl reaction, with conditions otherwise as described above. Genomic *RYR1* PCR products were sequenced using the RYR1-14.Forward primer at the Advanced Genetic Analysis Center of the University of Minnesota.

Polymerase Chain Reaction Restriction Enzyme Digestion

The candidate mutation creates a recognition sequence (5'. . . TGG**C**CA . . .3') for the restriction enzyme

‡‡ Additional information can be found at the following Web site: National Center for Biotechnology Information. Available at: http://www.ncbi.nlm.nih.gov/genbank. Accessed July 15, 2001

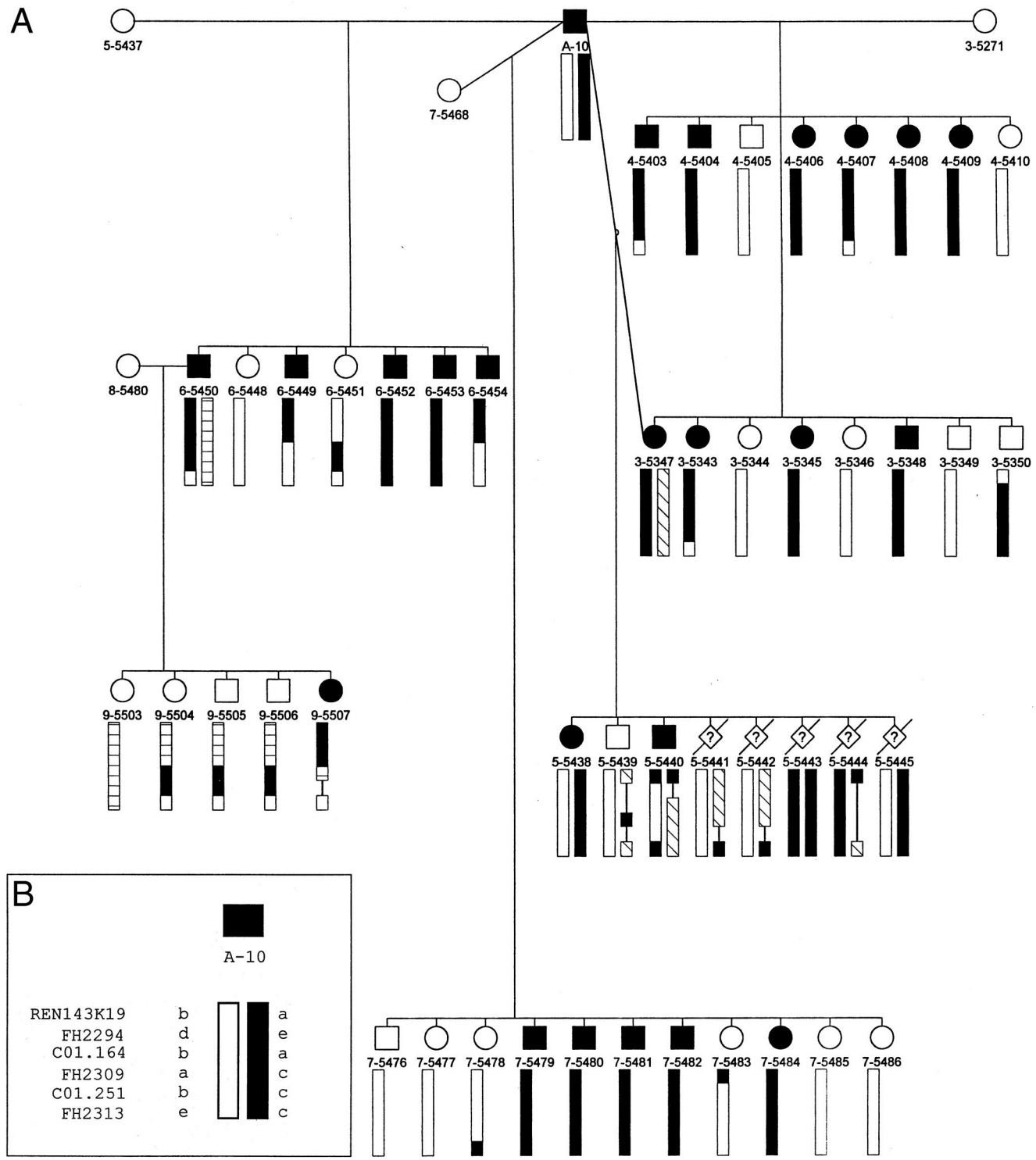

Fig. 1. The canine malignant hyperthermia (MH) colony. MH-susceptible (MHS) dogs are represented by black; MH-normal (MHN) dogs are represented by white; dogs of unknown phenotype are represented by a question mark in a diamond. No. A-10's haplotype is indicated in the box. Bars for the offspring are only shown for A-10's chromosome. MHS or MHN diagnoses were made by *in vivo* halothane–succinylcholine clinical challenge and *in vitro* contracture testing. Genotyping with CFA01 markers was performed as described in Materials and Methods.

*Msc*I (New England Biolabs, Beverly, MA), enabling its detection in the pedigree by PCR followed by enzyme digestion. PCR primers RYR1-14.Forward and RYR1-16.Reverse (table 1) were used to amplify the genomic segment containing exon 14 to exon 16 (product RYR1-1416) from all 52 colony dogs and from 24 unrelated dogs with no known history of MH or exercise intolerance. For each digestion, 7.5 μl of genomic PCR product

Table 2. Recombination Frequencies (θ) and LOD Scores for MHS and CFA01 Markers from the CRIMAP Program

	REN143K19		MHS		FH2294		C01.164		FH2309		C01.251	
	θ	LOD	θ	LOD	θ	LOD	θ	LOD	θ	LOD	θ	LOD
MHS	0.05	8.83										
FH2294	0.07	7.65	0.03	9.42								
C01.164	0.12	6.44	0.07	8.16	0.00	12.04						
FH2309	0.25	2.35	0.23	2.63	0.20	4.42	0.16	4.86				
C01.251	0.18	3.05	0.11	4.82	0.17	4.32	0.09	5.79	0.06	8.97		
FH2313	0.31	1.18	0.27	1.52	0.30	1.78	0.31	1.18	0.23	3.53	0.20	2.96

MHS = malignant hyperthermia susceptibility; CFA01 = canine chromosome 1.

was incubated with 1.5 units of *Msc*I in a total reaction volume of 20 μl containing 2.0 μl NEB buffer 4 (New England Biolabs, Inc.) at 37°C for 2 h. The digestion products were size-separated by electrophoresis in 2.5% agarose in Tris Acetate EDTA buffer and observed by staining with ethidium bromide.

Statistics

The pedigree depicted in figure 1 was constructed with Cyrillic2 software.[25] Subject genotypes were entered in Cyrillic2 and exported to MLINK[26] and CRIMAP[27] for analysis. The most probable mechanism of inheritance was estimated using a likelihood ratio comparing autosomal dominant and recessive descent. Linkage between MHS and each marker was tested in MLINK with parameters selected for autosomal dominant inheritance at full penetrance. Loci were ordered using the TWOPOINT option of CRIMAP for calculation of LOD scores and recombination fractions (θ) between CFA01 markers. BUILD and ALL options were used to resolve the most probable order of markers and MHS. This order was verified by comparing the log of the likelihoods of the original order of paired loci to their reversed order using the FLIPS_N option. The map diagram was prepared using MapCreator (AJD Computing).§§

Results

In Vivo *Halothane–Succinylcholine Challenge Test Results*

The premonitory sign of MH in this mixed-breed pedigree is hypercarbia and increased carbon dioxide production (78.5 ± 28 ml · min^{-1} · kg^{-1}; mean ± SD) occurring as early as 10 min after exposure to halothane and succinylcholine, followed by tachycardia (178 ± 29 beats/min) and hyperthermia (40 ± 1.69°C) within the first hour.[19] Dantrolene administration rapidly reverses biochemical and clinical evidence of the syndrome. Arterial blood lactate does not differ between challenged MHN and MHS dogs, nor is skeletal muscle rigidity a prominent feature of the triggered condition.

In Vitro *Contracture Test Results*

In vitro contracture test results for each fasicle from each dog can be found in the Web Enhancement. Mean contracture responses to 3% halothane of muscle fasicles from dogs that did not develop clinical MH during *in vivo* challenge showed less than 0.2 g tension in all cases (0.02 ± 0.05 g), whereas muscle from each of the MHS dogs exceeded 0.2 g isometric contracture (0.83 ± 0.43 g). Eighteen of 23 MHN dogs showed no contracture in any fasicle in the presence of 3% halothane. The mean CSC was less than 6.0 mM caffeine (3.12 ± 1.36 mM) in all 24 dogs that were MHS by *in vivo* challenge and 3% halothane IVCT. Nineteen of 23 dogs that were MHN by *in vivo* challenge and 3% halothane required a CSC of greater than 6.0 mM on mean (13.06 ± 5.51 mM); in four MHN dogs, the mean CSC was less than 6.0 mM (No. 3-5271: 4.40 mM; No. 3-5344, 3.69 mM; No. 3-5350, 5.62 mM; No. 7-5478, 5.20 mM). Limited amount and viability of tissue precluded halothane CSC testing in three MHN and two MHS dogs diagnosed by *in vivo* challenge and 3% halothane contracture. The mean halothane CSC of 17 of 20 MHN dogs was greater than 2.0 mM caffeine (3.10 ± 0.77 mM); in three dogs it was less (No. 3-5271, 1.73 mM; No. 9-5503, 1.79 mM; No. 9-5504, 1.99 mM). The mean halothane CSC was less than 2.0 mM in 21 of 22 MHS dogs (0.85 ± 0.39 mM), with the exception of dog No. 4-5403 (2.84 mM).

Of the 47 F1 and F2 offspring in the colony, 23 dogs were MHS, 19 were MHN, and 5 in the back-cross sibship were indeterminate (fig. 1). Thirteen MHS pups were males and 10 were females, suggesting that if a gender bias exists in the canine MHS syndrome, the magnitude of its effect is small. Both affected and unaffected dogs were found in each of the six litters, ranging from 1 of 5 dogs MHS in sibship No. 9 to 6 of 8 dogs MHS in sibship No. 4. Because the probability of two affected parents producing an unaffected offspring is zero under the assumption of recessive inheritance, presence of the MHN pup (No. 5-5439) within the back-cross sibship excludes the possibility of recessive inheritance in this pedigree, as ascertained by IVCT. Furthermore, a distri-

§§ Additional information can be found at the following Web site: Barris W: MapCreator. Available at: www.ajdcomputing.com/wes/mapcreator/. Accessed July 15, 2001

Table 3. Recombination Frequencies (θ) and LOD Scores for MHS and CFA01 Markers from the LINKAGE Program

	REN143K19		FH2294		C01.164		FH2309		C01.251		FH2313	
	θ	LOD	θ	LOD	θ	LOD	θ	LOD	θ	LOD	θ	LOD
MHS	0.05	8.24	0.05	8.9	0.05	7.9	0.25	2.4	0.1	4.5	0.3	1.4

MHS = malignant hyperthermia susceptibility; CFA01 = canine chromosome 1.

bution compatible with autosomal recessive descent would require that the unrelated dams of all litters be heterozygous for MHS, with a homozygous MHS sire. Rather, heterozygosity by random selection of dams is unlikely in light of the presumed rarity of the phenotype.

Genotype Results

CFA01 marker haplotypes in the canine MH pedigree are depicted in figure 1. The propositus #A-10 is heterozygous for markers REN143K19, FH2294, C01.164, FH2309, C01.251, and FH2313, with two, four, two, five, three, and seven alleles segregating in the family, respectively. The propositus haplotypes are a-e-a-c-c-c and b-d-b-a-b-e in marker order REN143K19-FH2294-C01.164-FH2309-C01.251-FH2313. For nonrecombinant dogs, the a-e-a-c-c-c propositus haplotype segregates with the MHS trait, whereas the b-d-b-a-b-e propositus haplotype segregates with MHN.

The MHS propositus is an ab heterozygote for the marker REN143K19, whereas his MHN mates are bb homozygotes. Each of the offspring showing the ab REN143K19 genotype were MHS, with the exception of MHN dog No. 7-5483, indicating a single recombinant event between REN143K19 and the MHS locus in this individual. Similarly, the e allele of FH2294 segregated with MHS in every instance but one. The exception is individual No. 3-5350, with evidence for recombination between the FH2294 marker e allele and the MHS phenotype. The MHS mother of the back-cross sibship (No. 3-5347) carried one copy of chromosome 1 with an FH2294 e allele linked to MH from the propositus. Her second chromosome 1 contained an FH2294 e allele unlinked to MH from her mother (No. 3-5271). The offspring of her pairing with the propositus at the MH locus are homozygous with both MHS-linked FH2294 e alleles from the propositus chromosome (No. 5-5443, 5-5444), heterozygous with the MHS-linked FH2294 e allele from either the father (No. 5-5440) or the mother (No. 5-5438, 5-5445), or homozygous for the unlinked FH2294 e allele from the mother and the d allele from the unlinked propositus chromosome 1 (No. 5-5439, 5-5441, 5-5442).

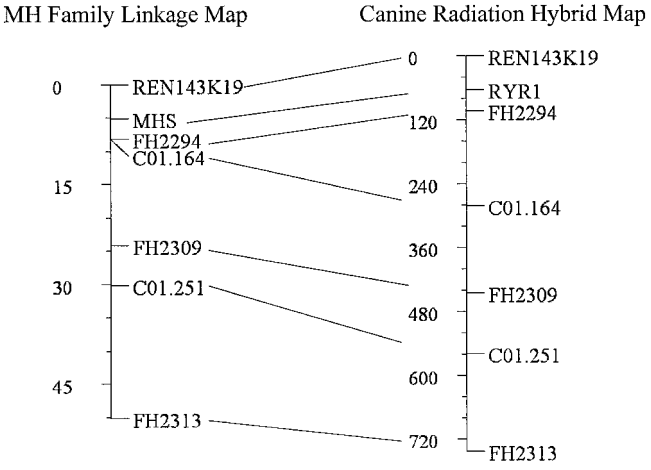

Fig. 2. A comparison of CFA01 maps constructed with linkage data from our malignant hyperthermia colony dogs and the whole genome radiation hybrid panel.[24] The map was made with the MapCreator program. The malignant hyperthermia (MH) colony linkage map represents our data measured in centiMorgans. The radiation hybrid map was assembled in accord with published distances measured in centiRays.[24] MHS = malignant hyperthermia susceptibility locus.

Within this panel of CFA01 markers, MLINK calculations reveal that FH2294 is most closely linked to MHS with a maximum LOD score at $\theta = 0.05$ of 8.9 (tables 2 and 3). Under the CRIMAP TWOPOINT option, MHS and FH2294 are linked with a maximum LOD score at $\theta = 0.03$ of 9.42. REN143K19 is linked to MHS at $\theta = 0.05$, LOD 8.24, and C01.164 at $\theta = 0.07$, LOD of 8.16, with no evidence for recombination between FH2294 and C01.164 in this pedigree. No other CFA01 markers were linked to MHS with a LOD greater than 3.0 at θ less than 0.1 in MLINK.

The BUILD and ALL options of CRIMAP were used to construct a genetic linkage map of CFA01 incorporating the MHS locus, presented for comparison with the published radiation hybrid map[24] in figure 2. Although primarily designed for use in mapping codominant loci, CRIMAP may also be of value for ordering expressed disease loci providing the genotypes are known. Only pups arising from an out-cross of an MHS to MHN subject are informative for CRIMAP analysis; failure to distinguish back-crossed MHS heterozygotes from MHS homozygotes precludes their entry into analysis. The outcrossed data were unable to resolve two possible orders: REN143K19-MHS-C01.164-FH2294-FH2309-C01.251-FH2313 *versus* REN143K19-MHS-FH2294-C01.164-FH2309-C01.251-FH2313. FLIPS_N analysis verified that both were equally likely, but that other than the ambiguous positions of markers FH2294 and C01.164, the remainder of the alignment is most probable. The finding that two flanking markers (REN143K19, FH2294) are recombinant for two different offspring (No. 7-5483, 3-5350) provides strong evidence that the mutant MHS locus is interposed. The MHS locus on the link-

age map is in the same proximal-telomeric position as the *RYR1* locus on the canine radiation hybrid map (fig. 2), and marker order is identical for both, although the linkage map was assembled from just 42 informative meioses in the MH pedigree, and distances between markers are therefore estimates.

Mutational Analysis Results

Two regions of the *RYR1* gene bracketing 21 human mutations associated with MHS[20] were selected for a canine mutation search by direct sequencing. Region I extends from nucleotides 22 to 1982, corresponding to amino acids 7–660 of the sarcoplasmic foot of the calcium release channel. Region II spans nucleotides 5946 to 7927, encoding amino acids 1982–2642. Analysis of reverse-transcription PCR product sequence from MHS dog No. 3-5348 disclosed a C for T polymorphism at nucleotide 1640 in exon 15, consistent with heterozygosity of the MHS genotype. The T1640C substitution was identified in replicate RNA isolations from the affected dog muscle, in replicate reverse-transcriptase PCR products from different No. 3-5348 cDNA templates, and in bidirectional sequencing of these PCR products. PCR products encompassing this region from No. 3-5348 were independently cloned and sequenced. The mutant T1640C allele was found in 6 of 22 clones, with 16 of 22 representing the wild-type T1640 allele, indicating that the polymorphism represents a genetic substitution rather than an error in amplification or sequencing.

The T1640C mutation results in the exchange of an alanine for valine at amino acid residue 547 in *RYR1* exon 15. The nucleotide sequences of canine regions I and II have been submitted to GenBank (accession No. A302128 and AF302129, respectively). Canine and human nucleotide sequence in regions I and II reveal more than 91% identity with more than 95% homology between derived protein sequences (table 4). A comparison of dog, human, rat, rabbit, and pig amino acid sequences surrounding the V547A site demonstrates that the valine 547 is phylogenetically conserved (fig. 3). Because the T1640C mutation creates a recognition site for restriction enzyme *Msc*I, each animal in the pedigree could be screened for the mutation using PCR–restriction fragment length polymorphism analysis. Figure 4 shows a representative agarose gel of *Msc*I digested PCR products for the three possible genotypes: the homozygous TT MHN wild-type (No. 4-5405) fragment is uncut

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                527
mhs dog         SLIRGNRSNC ALFSTNLDWL ASKLDRLEAS S
normal dog      SLIRGNRSNC ALFSTNLDWL VSKLDRLEAS S
human           SLIRGNRSNC ALFSTNLDWL VSKLDRLEAS S
rat             SLIRGNRTNC ALFSTNLDWL VSKLDRLEAS S
rabbit          SLIRGNRANC ALFSTNLDWV VSKLDRLEAS S
pig             SLIRGNRANC ALFSNNLDWL VSKLDRLEAS S
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Fig. 3. Amino acid comparison of exon 15 in dog and four other species. The malignant hyperthermia–susceptible mutation is highlighted. The compared sequences begin at amino acid number 527 as indicated.

Table 4. Interspecies Homology of RYR1 Nucleotide and Protein Sequence to That of the Dog

	Nucleotide (%)	Protein (%)
Region I		
Human	92	97.5
Pig	93	97.6
Rabbit	91	97
Region II		
Human	91.3	95.8
Pig	92	97.3
Rabbit	89.7	95.9
Exon 15		
Human	91	100
Pig	86.5	93.5
Rabbit	86.5	93.5
Rat	85.4	96.8

The MH-associated V547A mutation is in region I, exon 15.
RYR1 = ryanodine receptor; MH = malignant hyperthermia.

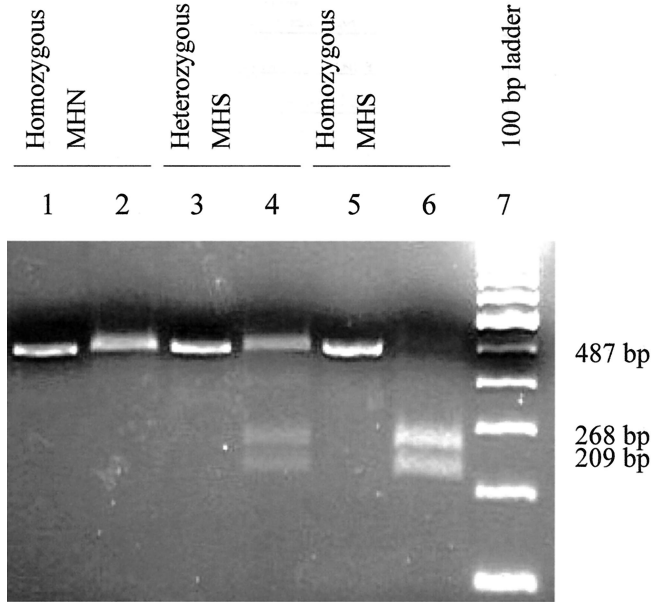

Fig. 4. Polymerase chain reaction–restriction fragment length polymorphism analysis of homozygous wild-type (No. 4-5405), heterozygous malignant hyperthermia–susceptible (MHS; No. 4-5404), and homozygous mutant (No. 5-5443) pups. Primers RYR1-14.F and RYR1-16A.R were used to amplify the 487–base pair (bp) fragments, which are shown uncut in lanes 1, 3, and 5 from left to right. Lane 7 is a 100-bp ladder. The MHS mutation creates a new *Msc*I recognition site, enabling analysis for the mutation. If the dog has two copies of the normal allele at position 1640, the digest will show only the 487-bp product (lane 2). If the dog has a single mutant allele at position 1640, the polymerase chain reaction product will have one allele digested and the other not, to show three size products on the gel; 487 bp, 268 bp, and 219 bp (lane 4). If the dog is homozygous for the MHS mutation no 487-bp fragment will be seen, with the polymerase chain reaction products from both chromosomes fully digested to the 268-bp and 219-bp fragments (lane 6).

at 487–base pair in length; the homozygous CC mutant (No. 5-5443) product is fully digested to 268 and 219–base pair fragments; all three fragment sizes are present in the digested PCR products of MHS dogs heterozygous for the T1640C substitution (No. 4-5404). Every dog diagnosed as MHS by the IVCT showed the mutation. All dogs diagnosed as MHN by IVCT and *in vivo* challenge had two copies of the normal T1640 allele, including MHN dog No. 3-5350, which was recombinant between the MH locus and the FH2294 e allele, and MHN dog No. 7-5483, which was recombinant between the MH locus and the REN143K19 a allele. In this canine MHS pedigree, at $\theta = 0.0$, the maximum LOD score of the T1640C mutation with MHS is 12.29. None of 24 unrelated control dogs representing 48 chromosome 1 genotypes carried the T1640C substitution.

Discussion

Malignant hyperthermia in a dog was first reported in 1973.[1] No subsequent investigation has tested for linkage of the canine MH phenotype to candidate genes or anonymous DNA markers. Here we report that canine MH ascertained by *in vivo* halothane–succinylcholine clinical challenge and IVCT is tightly linked to markers in the near vicinity of *RYR1*, with a recombinant for each of the two markers most closely flanking the MHS–*RYR1* locus. Moreover, an intragenic T1640C substitution in *RYR1* exon 15, which generates a V547A mutation in the RYR1 peptide, is in perfect cosegregation with inheritance of the MHS trait. These findings, together with the absence of the V547A transition in a randomly selected group of control dogs, and previous recognition of the region as a mutational hotspot in human MH, suggest that the V547A mutation causes MH in this mixed-breed dog colony.

Although MH-like events during exposure to anesthetics in dogs have been documented for nearly 30 yr, in most of the previous reports monitoring was scant, clinical descriptions were incomplete, and IVCTs were either not performed or were conducted according to nonstandard protocols. Nevertheless, the diagnosis of MH based on clinical event, laboratory exposure to trigger anesthetics, or IVCT appears clear and convincing in a purebred Pointer,[1] Greyhounds,[2,3] mixed-breed Doberman Pinscher–German Shepherds,[5,6] a Labrador Retriever,[7] and a dog of unspecified breed.[4] Possible perioperative MH-like events have also been reported in a St. Bernard,[11] Greyhounds,[13] a Springer Spaniel,[15] and unspecified breeds,[10] but the available data are less persuasive for a diagnosis of true MH. In aggregate, the main features of the canine MH syndrome shared with humans and pigs include identical pharmacologic triggering agents, hypercarbia, hyperthermia, tachycardia, therapeutic efficacy of dantrolene and supportive measures, and death if untreated. Consistent with our observations, but in contrast to manifestations of the disorder in humans and pigs, lactic acidemia, metabolic acidosis, and extensor rigidity are absent in most cases and delayed in the remainder. Investigations comparing exercise performance in the normal dog and pig reveal superior canine oxidative metabolic capacity, cardiac output, acid-base and heat regulatory elements that may contribute to physiologic compensation during an acute trigger in a predisposed dog.[28] In turn, the more subtle canine MH presentation may underlie missed diagnoses in the past and failure to investigate the true incidence of MH in canine surgery to the present, particularly in the absence of continuous core temperature and end expiratory gas monitoring.

Despite the relative lack of rigidity that characterizes the canine MHS syndrome, precision of the IVCT for phenotypic assignment is well preserved across species boundaries. Indeed, the lower end of the range of mean generated contracture tension in 3% halothane customarily selected for human diagnosis[22] (*i.e.*, 0.2 g) is sufficient to resolve the phenotype of every individual in our colony in comparison both to *in vivo* halothane–succinylcholine challenge, and to *RYR1* mutational analysis. Parsimony of IVCT results between susceptible and nonsusceptible dogs and between susceptible and nonsusceptible pigs and humans points not only to a shared molecular pathology, but reaffirms the value of the IVCT as conventionally configured for establishing MHS genotype–phenotype correlations in future model organisms. Coupled with the results of controlled clinical exposure to trigger anesthetic agents, we believe these IVCT results leave little doubt that the phenotype under present investigation represents a true homolog of MH in a third species, thereby warranting molecular investigations.

Although dogs also display stress or exercise-induced hyperthermia,[12,14–16] with many features in common with porcine stress syndrome, nonanesthetic triggers in MHS humans are extremely rare. Porcine stress syndrome is triggered in MHS strains of pigs by exertion, excitement, hypoxia, or high ambient heat corresponding to an MH trigger in the absence of exposure to anesthetics. In pigs, porcine stress syndrome is often fatal, whereas in dogs exercise-induced hyperthermia is rapidly reversible. In one Greyhound, a nonstandard, uncontrolled IVCT performed after recovery from canine stress syndrome was judged to be normal,[17] whereas in a Springer Spaniel the IVCT was positive.[15] No episodes of exercise intolerance or exertional hyperthermia have been observed in the MHS members of our colony. Species-specific differences in the fiber type composition of skeletal muscle may account, in part, for disparate susceptibilities to nonanesthetic stress syndromes. Human and pig muscle consists of fiber types I, IIA, IIB, and IIC.[29] Absence of the more highly glycolytic

type IIB fibers in dog skeletal muscle[30] may account for decreased lactate accumulation, minimal rigidity during an anesthetic MH trigger, and the reduced incidence and rapid recovery from nonanesthetic exercise-induced hyperthermia. As a consequence of our genetic investigations, it will now be possible to test dogs with exercise-induced hyperthermia or canine stress syndrome for a casual MHS mutation with the aim of unraveling potential associations between the clinical disorders.

For more than 100 millennia, the dog has been under intense breeding pressure in selection of traits conjoined to the hunt.[31] Paradoxically, it is the working and sporting breeds that appear to be at greatest risk for MH susceptibility. More than 300 modern strains of dogs originating from a progenitor wolf pool[32] share many of the traits and disorders that afflict humans, providing exceptional models for the investigation of genetic contributions to cellular physiology and pathology.[33] Very recently, canine genetic linkage and radiation hybrid maps using polymorphic microsatellite markers were developed in support of the Dog Genome Project.[23,24]‖‖ These reagents, in tandem with rigorous diagnosis and controlled mating based on *a priori* determinations of the MHS phenotype, enabled our identification of the canine MHS locus as the *RYR1* gene on dog chromosome 1 (CFA01).

The sarcoplasmic reticulum ryanodine receptor (RYR1) (calcium release channel), and the closely opposed transverse tubule dihydropyridine receptor (voltage-dependent calcium channel CACNA1S) are key components of the excitation–contraction coupling apparatus in skeletal muscle. Normal muscle contractility commences with dihydropyridine receptor α_1 subunit conformational changes during t-tubule depolarization coupled to gating of RYR1, and release of sarcoplasmic reticulum calcium stores into the myoplasm. The calcium release channel is a large (564,000 Da) homotetrameric protein with high and low affinity sites for the binding of ryanodine associated with open channel conductance and inactivation, respectively. Halothane, other volatile anesthetic agents, and caffeine release calcium from the sarcoplasmic reticulum membrane by activating the channel.[34] Succinylcholine elicits MH by indirect action on the t-tubule–sarcoplasmic reticulum triad secondary to nonphysiologic depolarization at the surface neuromuscular junction. In MHS individuals, RYR1 opens for a longer interval than normal in the presence of triggering drugs, and excessive calcium is extruded into the sarcoplasm.[35] Elevated resting sarcoplasmic calcium concentrations activate muscle contraction, promoting adenosine triphosphate hydrolysis, accelerated metabolism, and hyperthermia, which typify an MH reaction.[35]

Human *RYR1* consists of a 15,393–base pair cDNA transcript, with 106 exons spanning 158 kb of genomic DNA, encoding 5,038 amino acids, making it one of the largest genes known.[36] Close interspecies similarity in the regions of *RYR1* we have sequenced suggests that the dog skeletal muscle *RYR1* homolog will be very similar in overall size and structure to the human gene (table 4). Linkage of MHS to *RYR1* in pigs and humans was first reported in 1990,[34] with porcine MH traced to a *RYR1* R615C mutation shortly thereafter.[37] In humans, 21 mutations encoding the sarcoplasmic "foot" region of the RYR1 protein near the amino terminus (region I) or central portion (region II) are thought to cause MHS or central core disease, with one MHS mutation near the C terminus.[20] Mutations in the α_1 subunit of the dihydropyridine receptor (*CACNA1S*)[38] and in carnitine palmitoyl transferase II[39,40] have also been associated with MH in humans lacking a coexisting clinical myopathy. Thus, porcine MH is genetically homogenous with no evidence for a second gene or mutation, whereas human MH is a genetically heterogeneous syndrome arising from alterations in *RYR1*, *CACNA1S*, and other components of skeletal muscle calcium regulation. Because the current investigation is the first to report canine MH linkage, it is premature to speculate whether dog MH will be genetically homogenous or heterogeneous.

A second feature differentiating MH in humans and pigs is the mechanism of inheritance. Porcine MH is invariantly transmitted as an autosomal recessive trait. In humans, clear-cut autosomal dominant MHS has been described in a number of large pedigrees, although the majority of families with an MH proband are too small and inadequately characterized to specify with certainty. Inheritance of MHS in our colony of mixed-breed dogs, with a single mutant copy of the RYR1 locus sufficient to confer the fully penetrant MHS phenotype, more nearly resembles the human syndrome in this regard. Predisposition to trigger on stress, and on first exposure to contraindicated anesthetics, suggests that the causal mutation is more disruptive of RYR1 function in the pig than in the human or dog. However, the opposite is more likely to be the case since a single mutant copy is incapable of manifesting the MHS recessive clinical trait in pigs, nor is the porcine MHS heterozygote reliably detected by IVCT. Because heterozygotes pass MHS to 50% of their offspring, canine MH is not restricted to single strains showing a preponderance of recessive disorders sustained by highly ordered matings. Rather, all dog breeds are at potential MHS risk.

In our pedigree, all MHS dogs by phenotype carry the T1640C genotype, and all MHN dogs lack the mutation. Taken alone, these data are presumptive for causality of the MHS trait, but it is also plausible that the *RYR1* T1640C substitution merely serves as a proxy polymorphism for a truly causal mutation elsewhere in *RYR1* or in a nearby gene. Evidence against the latter hypothesis

‖‖ Additional information can be found at the following Web site: The FHRC Dog Genome Project. Available at: http://www.fhcrc.org/science/dog_geome/dog/html. Accessed July 15, 2001

includes linkage of the MHS trait to anonymous markers flanking *RYR1*, perfect cosegregation of MHS with T1640C incorporating two flanking recombinants, absence of the polymorphism in a randomly selected sample of control canine chromosomes, a consequent nonsynonymous amino acid substitution, causality of *RYR1* mutations for MHS in at least two other species, and occurrence of the mutation in a phylogenetically conserved region of *RYR1* known to be a mutational "hotspot" for MHS in humans. Additional support for causality of the V547A mutation awaits detailed analysis of its functional correlates compared with normal in RYR1 binding assays, single-channel recordings, and whole cell expression of the mutant protein. Inevitably, these investigations will also yield a deeper understanding of E-C coupling and its disorders in a second laboratory organism.

In conclusion, MHS in a large, well-characterized canine pedigree segregates in perfect accord with inheritance of a V547A mutation in the *RYR1* gene. These data represent both the first pharmacogenomic and the first autosomal dominant syndrome found in humans and dogs to share a common molecular mechanism. The presence of the specific mutation may now be sought in other species and dog breeds showing MH and related hyperthermic stress syndromes. Detection of the mutant allele by clinical challenge and IVCT suggests that the dog may be a useful model for the development of improved phenotypic detection of heterozygotes needed for human diagnosis and genetic investigations.

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