Exclusion of linkage of the RYR1, CACNA1S, and ATP2A1 genes to recurrent exertional rhabdomyolysis in Thoroughbreds

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Objective—To determine whether there was genetic linkage between the recurrent exertional rhabdomyolysis (RER) trait in Thoroughbred horse pedigrees and DNA markers in genes (the sarcoplasmic reticulum calcium release channel [RYR1] gene, the sarcoplasmic reticulum calcium ATPase [ATP2A1] gene, and the transverse tubule dihydropyridine receptor-voltage sensor [CACNA1S] gene) that are important in myoplasmic calcium regulation.

Animals—34 horses in the University of Minnesota RER resource herd and 62 Thoroughbreds from 3 families of Thoroughbreds outside of the university in which RER-affected status was assigned after 2 or more episodes of ER had been observed.

Procedures—Microsatellite DNA markers from the RYR1, ATP2A1, and CACNA1S gene loci on equine chromosomes 10, 13, and 30 were identified. Genotypes were obtained for all horses in the 4 families affected by RER, and data were used to test for linkage of these 3 loci to the RER phenotype.

Results—Analysis of the RYR1, CACNA1S, and ATP2A1 microsatellites excluded a link between those markers and the RER trait.

Conclusions and Clinical Relevance—It is likely that the heritable alterations in muscle contractility that are characteristic of RER are caused by a gene that is not yet known to cause related muscle disease in other species. (Am J Vet Res 2006;67:1395–1400)

Exertional rhabdomyolysis in horses is characterized by episodes of muscle cramping, stiffness of gait, sweating, reluctance to move, and muscle-cell damage that develop after mild to moderate exercise. Recurrent exertional rhabdomyolysis, a recurrent form of ER, is likely the most prevalent muscle disease affecting Thoroughbred horses. Results of epidemiologic studies conducted at a Midwestern US racetrack and in the United Kingdom indicate that 5% to 10% of all Thoroughbreds develop ER at some point during a racing season, up to 73% of trainers have at least 1 horse with RER, and recurrence is so frequent in 17% of affected horses that they do not race again that season. Evidence for a heritable basis of RER was reported by MacLeay et al in 1999. Markov-chain Monte Carlo analysis was used to determine the conditional probability for foundation genotypes and yielded evidence that the RER trait had an autosomal dominant mode of inheritance with variable expression. This suggests that foals of an RER-affected stallion or mare would have at least a 50% chance of inheriting the RER gene.

Results of recent studies suggest that a defect in regulation of muscle contractility is associated with RER. Muscle fiber bundles from affected horses have increased sensitivity to potassium-, caffeine-, and halothane-induced contracture in vitro, compared with muscles from control horses. Furthermore, myotubes (developing muscle fibers) derived from muscle biopsy specimens from affected horses respond to caffeine with a greater increase in myoplasmic calcium concentration than do myotubes from control horses, and the muscle relaxant dantrolene ameliorates muscle damage in exercising Thoroughbreds with RER. More recently, results of a standardized halothane-caffeine in vitro contracture test used in a controlled breeding trial indicated that the segregation of RER is consistent with an autosomal dominant trait. Genes encoding proteins that function in muscle-cell calcium regulation and excitation-contraction coupling are, therefore, logical candidate genes for causing RER.

During skeletal muscle excitation-contraction coupling, depolarization of the cell surface membrane triggers transverse tubule voltage sensors to open sarcoplasmic reticulum calcium channels, providing myoplasmic calcium for activation of contractile activity. Relaxation is mediated by resequestration of cal

ABBREVIATIONS

 ER Exertional rhabdomyolysis
 RER Recurrent exertional rhabdomyolysis
 MH Malignant hyperthermia
 LOD Logarithm of the odds
 NCBI National Center for Biotechnology Information
 BAC Bacterial artificial chromosome

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cium by sarcoplasmic-reticulum–associated calcium ATPase. Mutations in the sarcoplasmic reticulum calcium release channel (RYR1) gene, calcium ATPase (ATP2A1) gene, and transverse tubule voltage sensor (CACNA1S) gene cause heritable disorders such as MH and Brody disease, conditions typified by alterations in muscle calcium regulation and, in most cases, by increased sensitivity to in vitro contracture such as that observed in muscle specimens from Thoroughbreds with RER. In particular, several forms of ER in humans with abnormal in vitro contracture sensitivity are associated with mutations in the RYR1 gene, and an RYR1 mutation in 2 Quarter Horses that had fatal episodes of MH was recently reported.

The purpose of the present study was to determine whether the RYR1, ATP2A1, or CACNA1S genes were associated with RER in Thoroughbreds. Microsatellite markers within or close to those genes on the equine chromosomes were used to test for linkage with the RER trait in horses from the University of Minnesota RER Resource herd and in pedigrees of affected horses collected from several sources around the United States.

Materials and Methods

Resource populations of affected horses—A University of Minnesota RER resource herd has been described. Briefly, 3 Thoroughbred mares and 1 Thoroughbred stallion with a history of multiple clinical episodes of RER were mated, and subsequently, 5 mares with RER (including 2 of those previously mentioned) were repeatedly mated to 2 unaffected non-Thoroughbred stallions. An RER-affected stallion (the son of one of the original RER-affected mares) was bred to 6 unaffected mares. Diagnosis of RER susceptibility in parents in the university herd was also established with a validated in vitro test with the RER trait in horses from the University of Minnesota RER Resource herd and in pedigrees of affected horses collected from several sources around the United States.

The SLINK program was used to test the statistical power for detection of linkage in the 4 families. The mode of inheritance was defined as fully penetrant autosomal dominant, with a frequency of 0.05 in the Thoroughbred breed.

The distance between a microsatellite marker and the RER locus was defined as 3 cm (L = 0.05), and there were 3 alleles of equal frequency/marker. The mean LOD score derived from 200 simulations for the 4 families combined was 4.32 for a marker 5 cm from the RER gene (Table 1). Ninety-seven and a half percent of the simulations resulted in an LOD score > 1.00, 87.0% of the simulations resulted in an LOD score > 2.00, and 68.3% of the simulations resulted in an LOD score > 3.00. The university Thoroughbred family and one external family of Thoroughbreds were the most statistically powerful families for detecting linkage.

RYR1 and CACNA1S cDNA sequencing—Skeletal muscle mRNA was isolated from 4 RER-affected horses and 3 unaffected horses from the university RER herd, and cDNA was generated with commercially available kits. Reverse transcription PCR was used to generate over 4,000 bp of the equine RYR1 cDNA sequence (NCBI accession Nos. DQ318131 and DQ318132) and over 800 bp of the equine CACNA1S cDNA sequence (NCBI accession Nos. DQ318133 and DQ318134) by use of primers from conserved areas of the human and dog RYR1 and CACNA1S sequences. Comparison of those 4 equine cDNAs with human counterparts in the NCBI database by use of BLAST resulted in E values < 8e-125. The cDNA sequences of RER and control horses were examined for polymorphisms with a sequence analysis program.

Identification of microsatellite markers RYR1ms1 and CACNA1Sms1—The partial equine RYR1 and CACNA1S cDNA sequences allowed the design of primers (5'-GAGGC-CCTGGAGAAAAC-3' and 5'-GAGGCTGATGTGAGCG-CAC-3') to amplify across intron 45 of the equine RYR1 gene and primers (5'-TCCTAACCAGCCTGTGTTT-3' and 5'-GTGTTCCTCCTGCGTGTTG-3') to amplify across intron 13 of the equine CACNA1S gene. The equine RYR1 and CACNA1S genomic DNA sequences derived from those primers were assigned NCBI accession numbers DQ318780 and DQ318781, respectively. Comparison of those 2 equine DNA sequences with their human counterparts via BLAST resulted in E values < 4e-25. The same PCR primers were used to screen the CHORI-241 equine BAC library and identify BAC clones that contained the RYR1 and CACNA1S genes (clones 121M16 and 47B11, respectively). Deoxyribonucleic acid sequences of PCR products from those BAC clones matched those originally obtained from genomic DNA. A PsiI 1 subcloned library in phIndex vector was added to each forward primer, and individual clones were arrayed in 384-well plates. Filters containing the arrayed colony DNA were hybridized with a Ca10 probe, and positive colonies were sequenced to identify microsatellites RYR1ms1 and CACNA1Sms1, which are provided in NCBI accession numbers DQ318782 and DQ318783.

Mapping the RYR1 and CACNA1S genes—Polymerase chain reaction primers to amplify microsatellites RYR1ms1 and CACNA1Sms1 were summarized (Appendix). An additional 18-bp 5' tail sequence was added to each forward primer. Each primer pair was amplified by means of PCR across the Newmarket reference family in 12-μl reactions including 2 μl of a 1:20 dilution of DNA, 1.2 mM MgCl2, 1 pmol of the forward-tailed primer and 5 pmol of the reverse primer, 5 pmol of a fluorescent dye–labeled primer complement, 100 μM dNTP, and 0.5 units of Taq DNA polymerase. Polymerase chain reaction amplification reactions proceeded for 20 minutes at 95°C with 40 cycles of 30 seconds at 94°C, 30 seconds at 56°C, and 30 seconds at 72°C and a final extension of 15 minutes at 72°C. Polymerase chain reaction products were prepared for capillary gel electrophoresis according to manufacturer's recommendations and run on a capillary electrophoresis DNA fragment analyzer with fluorescence detection. Genotype data were analyzed with instrument software, and alleles were

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manually confirmed. The RYR1ms1 and CACNA1Sm1 genotypes were analyzed for linkage against all other markers genotyped in the Newmarket family with CRI-MAP.23

Analysis of microsatellite markers for linkage to RER—Polymerase chain reaction primers to amplify microsatellite markers near or within the candidate genes in the RER Thoroughbred resource families were summarized (Appendix 1). The RYR1ms1 and CACNA1Sm1 primers were the same as those used to genotype the Newmarket reference family, whereas the ATP2A1 primers that amplify an intronic microsatellite were those described by Blechynden et al.20 In addition, 3 microsatellites flanking the CACNA1S gene (UMNe088, UCDEQ455, and AHT023) were also genotyped and analyzed in the pedigrees of the horses with RER. Polymerase chain reactions and genotyping of each of 96 individuals (including 34 horses with RER) selected from the RER resource herd pedigrees were performed similarly as those described for the Newmarket reference family. Genotype data were analyzed via parametric 2-point analysis by use of the FASTLINK program.22 Logarithm of odds scores > 3.0 were considered as evidence of linkage, and LOD scores < -2.0 were considered as excluding the possibility of linkage.22

Results

Evaluation of equine RYR1 and CACNA1S cDNA sequences—Four thousand fourteen base pairs of equine RYR1 cDNA sequence encompassing the 2 major MH mutation hot spots in the human RYR1 gene were obtained.4 One thousand nine hundred seventy-seven base pairs of equine cDNA sequence corresponded to bases 138 to 2,114 (amino acids 2 to 661) of the human cDNA sequence (accession No. NM_0000540), and 2,037 bp of equine cDNA sequence corresponded to bases 5,980 to 8,016 (amino acids 1,949 to 2,628) of the human cDNA sequence. No differences were detected when the RER-affected and control horse RYR1 cDNA sequences were compared. Eight hundred twenty-two base pairs of equine CACNA1S cDNA sequence were obtained. This sequence corresponded to bases 2,102 to 2,623 and 3,361 to 3,662 of the human CACNA1S cDNA sequence (NM_000069), in which mutations responsible for human MH25 and hypokalemic periodic paralysis are located.26 No differences were detected when the RER-affected and control horse CACNA1S cDNA sequences were compared.

Genetic linkage mapping of RYR1ms1 and CACNA1Sm1—The Newmarket reference family genotype data enabled assignment of RYR1ms1 to the p-arm of Equus caballus chromosome 10 (ECA10). The nearest flanking microsatellites to RYR1ms1 that had previously been mapped on the Newmarket reference family were COR043 (maximum LOD = 25.29 at a value of 0.00), COR020 (maximum LOD = 22.92 at a value of 0.01), and TKY131 (maximum LOD = 33.01 at a value of 0.01). The Newmarket reference family genotype data also enabled assignment of CACNA1Sm1 to ECA30. The nearest microsatellite markers to CACNA1Sm1 that had previously been mapped on the Newmarket reference family were UMNe340 (maximum LOD = 26.19 at a value of 0.0), LEX075 (maximum LOD = 12.22 at a value of 0.02), UMNe088 (maximum LOD = 23.50 at a value of 0.02), and UCDEQ455 (maximum LOD = 9.43 at a value of 0.03).

Exclusion of linkage between RER and the candidate genes—Eight RYR1ms1 alleles were available in the RER resource pedigrees. Two-point linkage analysis with this marker excluded linkage (defined as LOD < -2.0) to the RER trait at all values from 0.0 to 0.10 in the university RER resource family (Table 2). Linkage to RER was also excluded at all values from 0.0 to 0.10 in the 3 external Thoroughbred families combined (LOD = -3.94). An LOD score of -4.68 at θ = 0.10 when all 4 RER families were combined strongly excluded the RER locus from this region of ECA10p that contains the RYR1 gene.

Microsatellite ATP2A1 had 5 alleles in our RER-affected horse populations. This marker excluded linkage between the ATP2A1 gene and RER at all values up to 0.10 in the university family of horses. However, it was able to exclude linkage to RER only at a value of approximately 0.01 in the 3 combined external pedigrees (Table 2). An LOD score of -3.12 at θ = 0.10 when all 4 RER families were combined strongly excluded the RER locus from this region of ECA13 that contains the ATP2A1 gene.

Only 3 alleles of the CACNA1Sm1 gene were detected in our RER pedigrees and linkage with the RER trait was not excluded at any value of in any individual family or when all 4 families were combined (Table 2). The lowest LOD score for CACNA1Sm1 in all 4 families combined was -1.03 at θ = 0.0.

Additional microsatellite markers from the CACNA1S region on ECA30 were genotyped on the university and external RER-affected populations. At a value of 0.05, UMNe088, the closest marker to CACNA1S that was genotyped in our RER pedigrees, had LOD scores for linkage to RER of -1.58 in the university herd, -3.66 in the combined external RER families, and -5.23 when data from all 4 RER families were combined (Table 3). The next closest marker to CACNA1Sm1 genotyped in our RER populations, UCDEQ455, which also had a value of 0.05, had LOD scores of 1.00, 2.00, and 3.00 when data from all families were combined are given in the upper portion of the table. The percentage of simulations that yielded LOD scores > 1.00, 2.00, and 3.00 when data from all families were combined are given in the lower portion of the table.

Table 1—Contributions of 4 resource families of Thoroughbreds to an overall LOD score for linkage to the RER trait estimated by simulated linkage. The simulated linkage variables were defined as a marker with 3 alleles at a recombination fraction (theta [θ]) of 0.05 with respect to the RER locus. The mean contribution of individual families and the 4 families combined are given in the upper portion of the table. The percentage of simulations that yielded LOD scores > 1.00, 2.00, and 3.00 when data from all families were combined are given in the lower portion of the table.

<table>
<thead>
<tr>
<th>Family</th>
<th>No. of horses</th>
<th>LOD score</th>
<th>contribution</th>
</tr>
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<tbody>
<tr>
<td>UMN herd</td>
<td>32</td>
<td>1.66</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>27</td>
<td>1.19</td>
<td></td>
</tr>
<tr>
<td>3</td>
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<td>4</td>
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</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>4.32</td>
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</table>

UMN = University of Minnesota RER resource herd.
scores for linkage to RER of –1.28 in the university RER family, –0.94 in the combined external RER families, and –2.26 in all 4 families combined. Lastly, at a θ value of 0.10, microsatellite AHT023 had an LOD score for linkage to RER of –1.37 in the university RER family, –1.74 in the combined external families, and –3.12 when all 4 families were combined. The UMNe088, UCDEQ455, and AHT023 markers, therefore, were not statistically linked to RER at θ values greater than their estimated distance from the CACNA1S locus.

**Discussion**

It is increasingly evident that many conditions affecting equine health have a genetic component and
that breeding practices that select for desirable traits may also inadvertently select for undesirable traits. Results of previous studies\(^1,13,14\) indicate that RER in Thoroughbreds is heritable, possibly as a dominant trait modifiable by environmental factors, and affects the health and performance of as many as 10% of all racehorses each season. The wide variety of remedies and treatments used in the management of RER reflects the scarcity of much-needed information regarding the physiologic and genetic bases of the disease. Elucidation of the molecular genetic basis for RER would allow development of genetic tests to decrease the influence of the disease on the population and better ways of diagnosing the disease and managing horses known to be genetically susceptible to the same form of ER.

Malignant hyperthermia is a heritable disorder of skeletal muscle calcium regulation in which susceptible individuals have muscle contracture and hypermetabolic responses to halogenated anesthetics in vivo and in which muscle biopsy specimens have high sensitivity to halothane- and caffeine-induced contracture in vitro.\(^22\) A large proportion of humans with MH have mutations in the \(Ryr1\) gene that lead to abnormal regulation of calcium release from the sarcoplasmic reticulum,\(^2,6\) thus explaining the characteristically high sensitivity to in vivo and in vitro muscle contracture. The possibility that RER is also caused by a defect in muscle calcium regulation is supported by the high sensitivity of muscle biopsy specimens from RER-affected Thoroughbreds to halothane- and caffeine-induced contracture.\(^4,7\) The possibility is further supported by the observation that ER in some humans (which also have abnormal in vitro contracture responses) is associated with \(Ryr1\) mutations\(^13,14\) and Thoroughbreds appear to have an unusually high incidence of postoperative rhabdomyolysis.\(^27\)

In the present study, several known \(Ryr1\) mutation hot spots were sequenced (including a recently reported MH polymorphism\(^15\) condition in Quarter Horses) in several RER-affected and control Thoroughbreds, but no sequence differences were detected. Nevertheless, it was still possible that mutations associated with RER existed elsewhere in the \(Ryr1\) gene. Accordingly, we used a microsatellite marker derived from a BAC clone that contained a segment of the equine \(Ryr1\) gene to test for genetic linkage between the \(Ryr1\) gene and RER. The population of horses in the 4 Thoroughbred families evaluated in the present study was statistically powerful enough for linkage to be detected if it existed. The \(Ryr1\)ms1 marker on ECA10p clearly excluded linkage up to \(\theta = 0.10\) in both the universe and external RER-affected populations. No abnormalities in \(Ryr1\) calcium channel function were detected in sarcoplasmic reticulum vesicles isolated from muscle of affected Thoroughbreds in 1 study\(^4,6\); however, because of the complexity of \(Ryr1\) regulation in vitro and in vivo, that finding did not preclude a role for this protein in RER. The genetic linkage data presented here exclude the \(Ryr1\) locus as conferring genetic susceptibility to RER in the populations of Thoroughbreds studied.

Other heritable disorders of muscle calcium regulation are caused by mutations in the \(Cacna1s\) and \(Atp2a1\) genes. One mutation in the \(Cacna1s\) gene is associated with a form of MH,\(^16\) whereas several mutations in the \(Atp2a1\) gene are associated with the delayed muscle relaxation associated with Brody disease.\(^11,12\) In the present study, microsatellite markers near those genes were also evaluated for the possibility of linkage to RER. An intragenic microsatellite marker excluded the possibility that the \(Atp2a1\) gene on ECA13 caused RER in the Thoroughbred families evaluated. Although the \(Cacna1s\)ms1 microsatellite derived from a \(Cacna1s\) BAC clone could not exclude linkage because of 1 prevalent allele, 3 flanking microsatellite markers on ECA30 did exclude linkage of that locus to RER at appropriate \(\theta\) values when all 4 RER pedigrees were combined.

Pedigrees from 4 RER resource families were used to detect or exclude linkage to microsatellite markers near candidate genes, raising the possibility that our study could be confounded if horses in the different families did not have the same form of RER. However, the Thoroughbred parents used to create the University of Minnesota herd came from various locations across the United States and were initially identified by means of the same clinical criteria used by veterinarians to diagnose RER in the external families. Analysis of the microsatellite markers at the \(Ryr1\) and \(Atp2a1\) genes permitted exclusion of linkage of those loci to RER when the external and internal equine families were analyzed separately. In addition, although the microsatellites at the \(Cacna1s\) gene locus could only exclude linkage to RER when all families were combined, none of those markers yielded positive LOD scores when the families were analyzed separately. Although this does not conclusively prove that all of our resource families had the same form of RER, there was no support for linkage of the candidate genes with RER.

In conclusion, Thoroughbreds with RER have an intrinsic heritable defect in regulation of muscle contraction that is observed in vitro as increased sensitivity of muscle biopsy specimens to potassium-, caffeine-, and halothane-induced contracture.\(^4,7\) Those results, coupled with the high concentrations of myoplasmic calcium observed upon exposure of cultured RER myotubes to caffeine,\(^1\) indicate that RER is caused by a defect in a gene that participates in skeletal muscle calcium regulation. Results indicated that certain important genes known to cause heritable defects in muscle calcium regulation do not contribute to RER in Thoroughbreds. Whole-genome genetic linkage analysis has the potential to assist not only in defining the basis of an important disease in Thoroughbred horses, but also in understanding the basis for novel forms of ER in humans and other species.

b. Micro-Fasttrack 2.0 kit, Invitrogen Corp, Carlsbad, Calif.
d. Sequencher, Gene Codes Corp, Ann Arbor, Mich.
e. CHORI-241 equine BAC library. Children’s Hospital Oakland...
References


Appendix

Polymerase chain reaction primers used to amplify equine microsatellite markers RYR1ms1, CACNA1Sm1s, and ATP2A1.

<table>
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<tr>
<th>Microsatellite marker</th>
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<th>Reverse primer</th>
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<td>RYR1ms1</td>
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<td>ATP2A1</td>
<td>5′-GAAGGGAGCAATGGACGGCGG-3′</td>
<td>5′-TTGCTTACACCTGACTG-3′</td>
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