Desmosomal gene evaluation in Boxers with arrhythmogenic right ventricular cardiomyopathy

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Objective—To sequence the exonic and splice site regions of the 4 desmosomal genes associated with the human form of familial arrhythmogenic right ventricular cardiomyopathy (ARVC) in Boxers with ARVC and identify a causative mutation.

Animals—10 unrelated Boxers with ARVC and 2 unaffected Labrador Retrievers (control dogs).

Procedures—Exonic and splice site regions of the 4 genes encoding the desmosomal proteins plakoglobin, desmoplakin, and desmoglein-2 were sequenced. Sequences were compared for nucleotide sequence changes between affected dogs and the published sequences for clinically normal dogs and between affected dogs and the control dogs. Base-pair changes were considered to be causative for ARVC if they were detected in an affected dog but not in unaffected dogs, and if they involved a conserved amino acid and changed that amino acid to one of a different polarity, acid-base status, or structure.

Results—A causative mutation for ARVC in Boxers was not identified, although single nucleotide polymorphisms were detected in some affected dogs within exon 3 of the plakoglobin gene; exon 3 of the plakoglobin gene; exons 3 and 7 of the desmoglein-2 gene; and exons 6, 14, 15, and 24 of the desmoplakin gene. None of these changed the amino acid of the respective protein.

Conclusions and Clinical Relevance—Mutations within the desmosomal genes associated with the development of ARVC in humans do not appear to be causative for ARVC in Boxers. Genome-wide scanning for genetic loci of interest in dogs should be pursued. (Am J Vet Res 2007;68:1338–1341)

Arrhythmogenic right ventricular cardiomyopathy is a familial cardiomyopathy characterized by progressive right (and sometimes left) ventricular and interventricular myocardial atrophy and fibrofatty replacement. Clinically, ARVC is characterized by ventricular tachyarrhythmias of right ventricular origin that can lead to sudden cardiac death, and in some individuals, progression to myocardial dysfunction and heart failure. Arhythmogenic right ventricular cardiomyopathy is a common cause of heart disease in Boxers, and affected dogs may develop syncope or signs associated with congestive heart failure or die of sudden cardiac death without overt clinical signs. The diagnosis of ARVC has also been made in other species, including cats and humans. The familial aspects of ARVC have also been studied in other species, including cats and humans. Because 4 of the reported genes that contain causative mutations encode desmosomal proteins, it has been proposed that ARVC is a disease of the desmosome. Desmosomes are multiprotein complexes located in the cell membrane that provide both structural and functional integrity to adjacent cells as well as a link between the plasma membrane to the cytoskeleton. Causative mutations have been identified in the 3 genes that encode desmoplakin, plakoglobin, and plakophilin-2; these desmosomal proteins are responsible for the mechanical coupling of the myocytes and provide a continuous cell-to-cell connection to the sarcomeric actin and the intermediate filaments. A fourth gene containing causative mutations is desmoglein-2. Desmogleins form one of the essential transmembrane components of desmosomes. The discovery of causative mutations for ARVC within genes encoding desmosomal proteins has led to the proposal that ARVC is

### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ARVC</td>
<td>Arrhythmogenic right ventricular cardiomyopathy</td>
</tr>
<tr>
<td>TGF-β3</td>
<td>Transforming growth factor-β3</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleoside triphosphate</td>
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The genetic evaluation of ARVC in humans has identified 9 different loci and 6 different causative genes. An autosomal dominant pattern of inheritance with variable penetrance is most common. Because 4 of the reported genes that contain causative mutations encode desmosomal proteins, it has been proposed that ARVC is a disease of the desmosome. Desmosomes are multiprotein complexes located in the cell membrane that provide both structural and functional integrity to adjacent cells as well as a link between the plasma membrane to the cytoskeleton. Causative mutations have been identified in the 3 genes that encode desmoplakin, plakoglobin, and plakophilin-2; these desmosomal proteins are responsible for the mechanical coupling of the myocytes and provide a continuous cell-to-cell connection to the sarcomeric actin and the intermediate filaments. A fourth gene containing causative mutations is desmoglein-2. Desmogleins form one of the essential transmembrane components of desmosomes. The discovery of causative mutations for ARVC within genes encoding desmosomal proteins has led to the proposal that ARVC is

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a result of defective cell-adhesion proteins in the desmosome of cardiomyocytes. The lack of normal protein or incorporation of mutant protein into the cardiac desmosome may provoke detachment of myocytes at the intercalated disks, especially under conditions of mechanical stress. This impaired cell adhesion could lead to accelerated apoptosis of myocardial cells, resulting in the underlying pathogenic mechanisms. Causative mutations for ARVC have also been identified in 2 genes that encode the non-desmosomal proteins TGF-β3 and the cardiac ryanodine receptor, respectively. Interestingly, although TGF-β3 does not encode a desmosomal protein, it is involved in modulation of cell adhesion.

Similarities between the human and canine forms of ARVC include myocardial atrophy and fibrofatty replacement predominantly in the right ventricle, ventricular tachyarrhythmias of right ventricular origin, and a clinical syndrome that can include sudden cardiac death or myocardial dysfunction, as well as an autosomal dominant mode of inheritance. By use of linkage analysis, our group has determined that the cardiac ryanodine receptor is not linked with ARVC in Boxers. The purpose of the study reported here was to sequence the exonic and splice site regions of the 4 desmosomal genes associated with the ARVC-affected Labrador Retrievers (control dogs) were evaluated. Criteria for diagnosis of ARVC included a clinical phenotype of right ventricular-origin ventricular premature complexes (> 1,000 ventricular premature complexes/24 h) and, when present, syncope, sudden cardiac death, or right-sided heart failure. Adult Labrador Retrievers were chosen as the control dogs because this breed is known to develop ARVC.

Materials and Methods

This study was conducted in accordance with the guidelines of the Animal Care and Use Committee of The Ohio State University College of Veterinary Medicine. Written consent authorizing study participation was obtained from each dog owner.

Dogs—Ten unrelated Boxers with ARVC and 2 unaffected Labrador Retrievers (control dogs) were evaluated. Criteria for diagnosis of ARVC included a clinical phenotype of right ventricular-origin ventricular premature complexes (> 1,000 ventricular premature complexes/24 h) and, when present, syncope, sudden cardiac death, or right-sided heart failure. Adult Labrador Retrievers were chosen as the control dogs because this breed is known to develop ARVC.

Procedures—Blood (5 mL) was collected via venipuncture of a jugular vein from each dog. Genomic DNA samples were prepared from whole blood samples as previously described. Briefly, cells were osmotically lysed in 2X sucrose-Tris-Tris-NH4Cl buffer, and nuclei were pelleted via centrifugation at 800 × g for 20 minutes at 4°C. Pellets were resuspended in saline (0.9% NaCl)-EDTA solution with 1% SDS and 50 μg of proteinase K/mL, and incubated overnight (approx 10 hours) at 56°C. The samples underwent 2 successive phenol-chloroform-isoamyl (25:24:1 [pH, 8]) and 1 chloroform extractions. Finally, the DNA was ethanol precipitated, washed with 75% ethanol, and resuspended in 250 μL of Tris-EDTA buffer (10 mM Tris-HCl and 1mM EDTA [pH, 8]).

Polymerase chain reaction amplification primers were designed for all exons by use of computer software and published canine nucleotide sequence information for plakophilin 2, plakoglobin, and desmoplakin (ENSCAFT00000016656, ENSCAFT00000008400, and ENSCAFT0000009570, respectively; Appendix). Amplification primers for the predicted sequence of desmoglein were designed by use of the published human sequence (XM_547622) and the published canine genome (Q14126).

Standard PCR amplifications were carried out by use of NH4SO4 amplification buffer, Taq DNA polymerase (0.1 U/μL of reaction volume), 2.5mM MgCl2, 12.5μM of each dNTP, 2.5mM of each PCR amplification primer, and 100 ng of template DNA. Samples were denatured for 5 minutes at 94°C followed by 40 cycles of 94°C for 20 seconds; 58°C for 30 seconds; 72°C for 30 seconds; and finally, 72°C for 7 minutes. The annealing temperature was optimized to accommodate the respective primer requirement.

Table 1—Single nucleotide polymorphisms detected within the canine plakophilin-2, plakoglobin, desmoglein-2, and desmoplakin genes in 10 Boxers with ARVC and 2 unaffected Labrador Retrievers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Exon</th>
<th>Altered codon</th>
<th>Associated amino acid</th>
<th>Heterozygous or homozygous alteration</th>
<th>Group with detected alteration</th>
<th>No. of dogs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plakophilin-2</td>
<td>3</td>
<td>CGA/AGA</td>
<td>Arginine (R125)</td>
<td>Both</td>
<td>Control ARVC-affected</td>
<td>1</td>
</tr>
<tr>
<td>Plakoglobin</td>
<td>3</td>
<td>CGC/GGT</td>
<td>Arginine (R217)</td>
<td>Both</td>
<td>Control ARVC-affected</td>
<td>1</td>
</tr>
<tr>
<td>Desmoglein-2</td>
<td>3</td>
<td>CCC/CCT</td>
<td>Proline (P97)</td>
<td>Both</td>
<td>Control ARVC-affected</td>
<td>1</td>
</tr>
<tr>
<td>Desmoglein-2</td>
<td>7</td>
<td>GAT/GAC</td>
<td>Aspartic acid (D318)</td>
<td>Heterozygous</td>
<td>ARVC-affected</td>
<td>2</td>
</tr>
<tr>
<td>Desmoplakin</td>
<td>6</td>
<td>ATC/ATT</td>
<td>Isoleucine (I218)</td>
<td>Heterozygous</td>
<td>ARVC-affected</td>
<td>1</td>
</tr>
<tr>
<td>Desmoplakin</td>
<td>14</td>
<td>ATC/ATA</td>
<td>Isoleucine (I525)</td>
<td>Heterozygous</td>
<td>ARVC-affected</td>
<td>3</td>
</tr>
<tr>
<td>Desmoplakin</td>
<td>15</td>
<td>ACG/ACT</td>
<td>Threonine (T605)</td>
<td>Both</td>
<td>Control ARVC-affected</td>
<td>2</td>
</tr>
<tr>
<td>Desmoplakin</td>
<td>24</td>
<td>GCA/GCG</td>
<td>Alanine (A1038)</td>
<td>Both</td>
<td>ARVC-affected</td>
<td>4</td>
</tr>
</tbody>
</table>
Residual amplification primers and dNTPs were removed from the PCR product via enzymatic treatment. Amplicons then underwent nucleotide sequence determination and analysis by use of a sequencer and a forward and reverse primer for each reaction for every sample.

The sequences were compared to identify nucleotide sequence changes between affected dogs and the published canine sequence (derived from a Boxer) and between affected dogs and the control dogs. Base-pair changes were considered to be possibly causative for ARVC if they were detected in any of the affected dogs. If a base-pair alteration was detected in any of the affected dogs, the alteration was evaluated to determine whether it changed a conserved amino acid and whether that amino acid was changed to one of a different polarity, acid-base status, or structure because these could be used as criteria for a causative change.

Results

The Boxer group included 6 spayed females (age range, 3 to 11 years; mean, 8 years) and 4 castrated males (age range, 6 to 9 years; mean, 7 years). Affected dogs had a mean of 22,690 ventricular premature complexes/24 h (range, 1,799 to 91,000). Four of the dogs (1 male and 3 females) had a history of syncope; the other 6 dogs had had no clinical signs, and the diagnosis had been made after an initial identification of the arrhythmia.

No differences in the exonic and splice site regions of the plakophilin-2, plakoglobin, desmoplakin, and desmoglein-2 genes between the affected Boxers and the control dogs or published Boxer sequence were detected. Single nucleotide polymorphisms were evident within exon 3 of the plakophilin-2 gene, exon 3 of the plakoglobin gene, exons 3 and 7 of the desmoglein-2 gene, and exons 6, 14, 15, and 24 of the desmoplakin gene (Table 1). None of these changed the amino acid of the protein.

Discussion

In the present study, analysis of the exonic and splice site nucleotide sequences of the 4 desmosomal genes associated with the human form of ARVC did not identify any mutations in the Boxers with ARVC. It is possible that a causative mutation exists in the promoter or untranslated regions of these genes in affected dogs; however, all of the known human ARVC mutations within these genes are located in exonic regions.3,10,11,25-27

The inability to identify a causative mutation for ARVC in the desmosomal genes of dogs evaluated in our study could be associated with the substantial genetic heterogeneity that characterizes this disease in humans.3,10,12,13,25-27 It is possible that a similar degree of genetic heterogeneity exists in dogs and that there is a desmosomal gene mutation in some affected Boxers but not in those included in our study. The investigation was limited to 10 affected Boxers, and it is possible that evaluation of a larger number of affected Boxers from different families might be necessary to identify mutations in desmosomal genes associated with ARVC. However, this seems unlikely given that the Boxer is a pure breed that has had a closed gene pool in the United States since 1904.28 It might therefore be more reasonable to assume that most of the affected dogs of this breed would share a unique mutation as a result of the founder effect within a closed population that has a limited number of founders. It is possible that the canine form of ARVC is attributable to a mutation that is common to that in affected humans but is in a yet to be identified gene. This possibility is supported by the fact that to date, only an estimated 50% of humans with ARVC have an identifiable genetic cause for ARVC.3 It is therefore quite possible that ARVC in Boxers corresponds to the human condition and is caused by a nucleotide sequence alteration in a locus that has not been identified in humans.3 Finally, it is possible that this arrhythmic disease in dogs is not a true match to ARVC in humans even though numerous clinical and pathologic studies3,14,13,17 have revealed similarities in the disease between these 2 species.

The nucleotide sequence data from the dogs in the present study were compared with the published canine sequence as well as with findings in 2 adult Labrador Retrievers that were used as a negative control group. The Labrador Retrievers were included in the study as control dogs because they represent a breed of dog for which there is no reported incidence of ARVC.29 Further, the published canine sequence is derived from an adult Boxer.29 Arrhythmogenic right ventricular cardiomyopathy is an adult-onset disease with variable penetrance in Boxers, and the published sequence could have been derived from an affected Boxer for which the diagnosis of ARVC had not been made.

Previously, a mutation in the cardiac ryanodine receptor gene (RYR2) was excluded as a likely cause for Boxer ARVC via linkage analysis.30 Further studies are needed to determine whether TGF-B3 plays a role in the development of ARVC in Boxers.

The familial nature of ARVC in Boxers and the severe implications for affected dogs have led to increased interest in reducing the prevalence of ARVC among Boxers through careful selection of only unaffected dogs for breeding purposes. The development of a DNA test that could be used to evaluate dogs before they reach reproductive maturity is key to the reduction of ARVC among Boxers. In the present study, a causative mutation for the disease was not identified by use of a candidate gene approach. Genome-wide scanning for genetic loci of interest should be pursued.

Appendix

Exon number and transcript length for the canine plakophilin-2, plakoglobin, desmoplakin, and desmoglein-2 genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>No. of exons</th>
<th>Transcript length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plakophilin-2</td>
<td>14</td>
<td>2,637</td>
</tr>
<tr>
<td>Plakoglobin</td>
<td>14</td>
<td>2,326</td>
</tr>
<tr>
<td>Desmoplakin</td>
<td>25</td>
<td>8,807</td>
</tr>
<tr>
<td>Desmoglein-2</td>
<td>14</td>
<td>3,325</td>
</tr>
</tbody>
</table>

a. Fermentas, Hanover, Md.
b. ExoSapIt, GE Healthcare Bio-Sciences Corp, Piscataway, NJ.
c. ABI Prism 377 Sequencer, Applied Biosystems, Foster City, Calif.
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