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Genome-wide association identifies a deletion in the 3' untranslated region of Striatin in a canine model of arrhythmogenic right ventricular cardiomyopathy:

Identification of Striatin deletion in canine ARVC

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Abstract

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a familial cardiac disease characterized by ventricular arrhythmias and sudden cardiac death. It is most frequently inherited as an autosomal dominant trait with incomplete and age-related penetrance and variable clinical expression. The human disease is most commonly associated with a causative mutation in one of several genes encoding desmosomal proteins.

We have previously described a spontaneous canine model of ARVC in the boxer dog. We phenotyped adult boxer dogs for ARVC by performing physical examination, echocardiogram and ambulatory electrocardiogram. Genome-wide association using the canine 50k SNP array identified several regions of association, of which the strongest resided on chromosome 17. Fine-mapping and direct DNA sequencing identified an eight base pair deletion in the 3' untranslated region (UTR) of the striatin (STRN) gene on chromosome 17 in association with ARVC in the boxer dog. Evaluation of the secondary structure of the 3' UTR demonstrated that the deletion affects a stem loop structure of the mRNA and expression analysis identified a reduction in striatin mRNA. Dogs that were homozygous for the deletion had a more severe form of disease based on a significantly higher number of ventricular premature complexes. Immunofluorescence studies localized striatin to the intercalated disc region of the cardiac myocyte and co-localized it to three desmosomal proteins, plakophilin- 2, plakoglobin and desmoplakin, all involved in the pathogenesis of ARVC in human beings.

We suggest that striatin may serve as a novel candidate gene for human ARVC.

Introduction

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a heritable cardiomyopathy characterized by myocardial fibrofatty replacement and ventricular tachyarrhythmias (Awad et al. 2008; Basso et al. 2009). In human beings, it is most frequently inherited as an autosomal dominant trait with incomplete and age-related penetrance and variable clinical expression. The human disease is most commonly associated with a causative mutation in one of several genes encoding desmosomal proteins and has been referred to as a disease of the desmosome (Awad et al. 2008; Basso et al. 2009; Herren et al. 2009). However, the identified genes do not explain all cases of ARVC (Basso et al. 2009). It has been estimated that only 30–40% of cases of ARVC have mutations in a known disease causing gene (Sen-Chowdhry et al. 2007, Sen-Chowdhry et al. 2010). This suggests that there may be other genes associated with the development of familial ARVC that have yet to be identified.

We have previously characterized canine ARVC in the boxer dog (Basso et al. 2004). Similarities of the canine disease to that of ARVC in human beings are numerous (Basso et al. 2004; Meurs et al. 1999; Baumwart et al. 2009). Canine ARVC is familial and appears to be inherited as an autosomal dominant trait with reduced penetrance (Meurs et al. 1999). The clinical presentation of the canine disease is characterized by frequent left bundle branch block morphology ventricular premature complexes which may lead to syncope or sudden cardiac death (Basso et al. 2004; Meurs et al. 1999). Magnetic resonance imaging identifies right ventricular dilation and aneurysms, reduced right ventricular ejection fraction, and bright anterolateral and/or infundibular ventricular signals consistent with fatty infiltrate (Basso et al. 2004; Baumwart et al. 2009). Pathologic analysis is characterized by right ventricular enlargement, aneurysms, and myocyte loss with replacement by fatty or fibrofatty infiltrate (Basso et al. 2004). Despite the significant similarities for ARVC between the two species, molecular evaluation of the most common desmosomal ARVC candidate genes for the human disease did not identify a causative mutation in the splice site or exonic regions of these genes in the dog (Meurs et al. 2007). The objective of this study was to identify the genetic alteration (s) associated with the development of ARVC in this canine model using genome wide association.

Methods

Selection of animal subjects

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 client.

As part of an ongoing study of the heritability of canine ARVC, three hundred pet boxer dogs over one year of age were prospectively recruited for participation. All dogs were evaluated with physical examination, electrocardiogram, echocardiogram, and a 24-hour ambulatory electrocardiogram using a 3-channel transthoracic system (Delmar Accuplus 363 Holter Analysis System, Irvine, CA). Pedigrees were collected when available.

Diagnostic criteria for canine ARVC included the presence of ≥ 500 ventricular premature complexes of right ventricular origin /24 hours (normal dogs have an average of 2/24 hours) and, when present, syncope (Meurs et al. 2001). Since all participating dogs were client owned pets only a small number of animals were available for more invasive studies including magnetic resonance imaging (MRI) and post mortem evaluation. Additional supportive findings for diagnosis including MRI for right ventricular enlargement and/or fatty infiltrate (15 dogs) and histopathologic analysis of the right ventricular myocardium demonstrating fibrofatty infiltrate in this group of dogs has been previously published (25 dogs) (Basso et al.

2004; Baumwart et al. 2009). Dogs with echocardiographic abnormalities suggestive of congenital heart disease or dilated cardiomyopathy were excluded. Using these criteria, 65 boxer dogs were diagnosed as ARVC-affected. This included 30 males (18 castrated, 12 intact) and 35 females (16 spayed, 19 intact) with an average age of 7 years.

Criteria for accepting a boxer dog as a control required a minimum age of 6 years, and a normal cardiovascular physical examination with < 100 ventricular premature complexes /24 hours.

One hundred canine DNA samples were also obtained from the laboratory bank of canine DNA collected from eleven different breeds of dogs as controls.

Genome-wide association analysis and fine-mapping

Five to seven milliliters of blood was collected from the jugular vein from each dog for genomic DNA extraction as previously described (Meurs et al. 2000).

Genome wide analysis (GWA) was performed with the Affymetrix Canine Genome 2.0 Array “Platinum Panel” (Affymetrix, Santa Clara, CA) containing 49,663 single nucleotide polymorphisms (SNP) markers with DNA from 46 boxer ARVC cases and 43 boxer controls. Only affected and control dogs with pedigrees were used for the GWA and participating dogs were specifically selected so that no dogs were related within a three generational pedigree. SNP genotypes were obtained following the human 500K array protocol, but with a smaller hybridization volume to allow for the smaller surface area of the canine array as previously described (Karlsson et al. 2007). Case-control GWA mapping was evaluated with PLINK (Purcell et al. 2007). Haplotype analysis was performed with Haploview (Barrett et al. 2004). Additionally, a joint association model was used to evaluate the association between ARVC and the deletion while accounting for the strongest markers on the chip.

Thirty-three additional canine SNPs (<http://www.broad.mit.edu/node/459>) surrounding the identified regions of interest (Chromosome 17:31,965,778–32,483,216 and Chromosome 17:35,115,477–37,841,551) were selected for fine mapping in a subset of 20 ARVC cases and 20 controls selected from the original group of dogs as described above. Polymerase chain reaction (PCR) amplification was used to amplify each SNP. Standard PCR amplifications were carried out with a cocktail of NH₄SO₄ amplification buffer, Taq DNA polymerase (0.1 units/μL of reaction volume), 2.5mM MgCl₂, 12.5μM of each dNTP, 2.5mM of each 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 72°C for 7 minutes. Annealing temperature was optimized to accommodate the respective primer requirement. Amplicons were analyzed on an ABI Prism 377 Sequencer (Applied Biosciences, Foster City, CA). Sequences from cases and controls were scored for alleles at each SNP. Allele frequencies were compared between case and control groups using Fischer’s Exact test with $p < 0.05$ considered significant.

Mutation detection

PCR primers were designed for exons and splice site regions of four candidate genes within identified regions of interest, solute carrier family 8 (sodium-calcium exchanger), member 1 (SCL8A1) (35,108,845–35,410,988), potassium channel, voltage-gated, subfamily G, member 3 (KCNG3) (37,142,239–37,189,465), Striatin (STRN) (32,375,918–32,435,652) and Vitrin (VIT) (32,288,868–32,346,341) as well as strongly conserved regions at the 5' and 3' untranslated regions (UTR) of each gene with Primer 3 software and the canine UCSC database (<http://genome.ucsc.edu/>) (Rozen et al. 2003). Standard PCR amplifications and sequencing was performed as described above. Sequences from cases and controls were compared to each

other as well as to the published normal CanFam2.0 canine sequence (<http://genome.ucsc.edu/>) to identify nucleotide sequence changes.

Subsequently, sequences from 59 cases (40/46 samples from GWA that had sufficient amount of remaining DNA), 40 controls (40/43 from GWA) and 100 non-boxer dog controls were genotyped for the eight base pair deletion at the 3'UTR region of Striatin.

Clinical correlation

Fifty nine cases were genotyped as either homozygous mutant, heterozygous, or homozygous wild-type for the deletion. The severity of disease (measured by the number of ventricular premature complexes (VPC) recorded/24 hours) was compared between the homozygous and heterozygous groups with an unpaired T test. $P < 0.05$ was considered significant.

Secondary structure and miRNA analysis

Prediction of local secondary structure was performed as described (Chen et al. 2006). Briefly, the 208 base pair wild-type sequence (comprising the 8 nucleotides deleted (CATAACACA) in the mutant allele plus 100 bases on either side) was evaluated with mfold (<http://mfold.bioinfo.rpi.edu>) to predict secondary structure. The analysis was then repeated with the same sequence but with the deletion removed.

Canine chromosome 17 was evaluated for possible miRNA binding sites in the 3' UTR of striatin with miRBASE (<http://www.mirbase.org/search.shtml>).

Expression analysis

Due to the difficulty of rapid collection of myocardial tissue immediately at time of death in client owned pet dogs that frequently die of sudden cardiac death associated with their disease, only samples from 4 ARVC affected boxers were of the quality needed for expression, protein and immunofluorescence analysis. Myocardial tissues were also available from two non-boxer healthy dogs (controls), and frozen at -80 degrees.

Quantitative real-time PCR was performed with right ventricular myocardial sections from 2 homozygous mutant Boxer dogs, 2 heterozygous Boxers, and 2 non-Boxer homozygous wild-type controls. Approximately 50 mg of myocardium was pulverized for total RNA extraction with the RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA). Reverse transcription was performed using Superscript II Reverse Transcriptase for cDNA synthesis (Invitrogen, Carlsbad, CA). Real-time PCR primers were designed for the exonic regions within KCNG3 (exons 1–2), SLC8A1 (exons 8–9) and STRN (17–18). Primers were also designed for regions spanning exons 6 and 7 of the hypoxanthine phosphoribosyltransferase (HPRT) gene to be used as a housekeeping gene. TaqMan Gene Expression Assays (Applied Biosystems) SYBR green mastermix (Qiagen) and real-time PCR protocols were used to amplify these targets using the Applied Biosystems 7500 Fast Real-Time PCR System. Samples were evaluated in triplicate. The triplicate CT values for each sample were averaged resulting in mean CT values for KCNG3, SLC8A1, STRN and HPRT. The SLC8A1, KCNG3 and STRN Ct values were standardized to the housekeeping gene by taking the difference $\Delta CT = Ct [Gene] - Ct [HPRT]$. Data was compared between control and affected dogs with a T test and are given as mean $\pm$ SEM of normalized gene expression levels. A $p < 0.05$ was considered significant.

Western blot

Frozen myocardial samples from the right ventricle of 2 non-Boxer controls and 2 heterozygous and 2 homozygous dogs were ground to a fine powder while cooled in liquid nitrogen and homogenized in Laemmli buffer. Protein concentration was determined with the Pierce 660 protein assay (Pierce Biotechnology, Rockford, IL, USA). Twenty micrograms of protein

extract for each dog was separated on a 4–20% gradient polyacrylamide gel and transferred to polyvinylidene fluoride membrane. Membranes were blocked with 5% milk and Striatin epitopes were probed by both a mouse monoclonal antibody generated against a WD repeat region (amino acid 450–600) of Striatin (1:100) (BD Transduction Laboratories, Franklin Lakes, NJ, USA) and a rabbit polyclonal antibody against a synthetic Striatin peptide which does not cross react with SG2NA (1:300) (Millipore). Blots were stripped and also probed with actin monoclonal antibody (1:100) (BD Transduction Laboratories) as a loading control. The species appropriate secondary IgG-HRP (1:10,000 dilution) (1:3,000) (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) was used followed by chemiluminescence detection and optical density determination using Quantity One software (BioRad, Hercules, CA, USA). Molecular weight was estimated using a standard curve generated from the Precision Protein Plus Western C standard (BioRad). Data was compared between affected and control animals with a T test. A $p < 0.05$ was considered significant.

Immunofluorescence confocal microscopy

Confocal microscopy of frozen myocardial sections (7 μ m) from the right ventricle of 2 homozygous mutant boxer dogs and 2 non-boxer homozygous wild-type controls was performed as described previously (Fidler et al. 2008). Briefly, myocardial sections mounted on glass slides were fixed in acetone at -20°C for 15 minutes, followed by air-drying for 30 minutes. The sections were rinsed with PBS and blocked in PBS with 2% normal goat serum, 1% bovine serum albumin and 0.2% Triton X-100 for 1 hour at room temperature. Sections were incubated with a rabbit polyclonal antibody for Striatin (1:50), (Millipore, Temecula, CA, USA) for 1 hour. Sections were then rinsed with PBS and incubated for 30 minutes at room temperature with corresponding secondary goat antibodies (1:100) (Invitrogen).

Co-localization was performed by incubating with mouse anti-Desmoplakin (1:200) (AbD serotec, Oxford, England), mouse monoclonal anti-Plakoglobin (1:500) (Sigma) or mouse monoclonal anti-Plakophilin -2 (1:200) (Acris Antibodies, Herford, Germany) primary and the appropriate secondary antibody. DAPI staining was performed to identify the nuclei. After the final rinsing step, sections were mounted with SlowFade Gold antifade reagent with DAPI to identify the nuclei (Invitrogen), coverslipped and examined using a Zeiss LSM 510 META confocal microscope (Carl Zeiss, Maple Grove, MN, USA).

Results

Genome-wide association mapping of canine ARVC identified a genome-wide significant association on chromosome 17 ($p_{\text{genome}} < 0.04$), as well as two lower peaks on chromosomes 11 and 26 (Figure 1A). The regions on 11 and 26 have not yet been investigated in detail as the available resources have been devoted to two chromosome 17 loci. One peak on chromosome 17 is a narrow peak from approximately 32.0–32.4 Mb (Figure 1B), with the most strongly associated SNP at chr17:32,194,234 ($p_{\text{raw}} = 2.4 \times 10^{-5}$ P-value corrected for genome-wide significance, $p_{\text{genome}} < 0.30$ based on 100,000 permutations using the software package PLINK). The second peak is a broader peak from 35–38.5 Mb, with the most strongly associated SNP at chr17:36,233,479 ($p_{\text{raw}} = 2.3 \times 10^{-6}$, $p_{\text{genome}} = 0.037$).

The associated region at ~36 Mb (36,225,013; 36,233,479) contains no defined genes. The closest flanking genes for this region were solute carrier family 8 (sodium-calcium exchanger), member 1 (SLC8A1) (35,093,791–35,410,988) and protein kinase domain containing, cytoplasmic homolog (PKDCC) (36,766,862–36,776,642). Both SLC8A1 and a nearby gene, potassium channel, voltage-gated, subfamily G, member 3 (KCNG3) (37,142,239–37,189,465) are known to be associated with cardiac function and were evaluated more thoroughly. Exons and promoter regions for SLC8A1 and KCNG3 as well as 2 highly evolutionarily conserved intronic areas for KCNG3 were sequenced in affected and unaffected

dogs, with no genetic differences identified. Additionally, expression analysis did not identify differences in expression levels for these two genes in right ventricular myocardial samples between control dogs and ARVC dogs. Ten evolutionarily conserved non-coding regions were selected based on the strongest LOD scores of the regions (<http://genome.ucsc.edu/cgi-bin/hgTracks>) as well as the immediate area surrounding the most informative SNPs (36,225,013; 36,323,479) and were sequenced. This included the following regions: 35,898,718–35,899,524; 36,224,542–36,224,721; 36,224,889–36,225,069; 36,231,570–36,231,832; 36,233,311–36,233,539; 36,237,800–36,238,022; 36,246,125–36,246,275; 36,645,296–36,645,894; 36,723,154–36,723,766; 36,767,371–36,767,582; 37,143,649–37,143,848 and 37,185,473–37,185,703, and again no sequence differences were identified between affected and unaffected dogs.

In the narrower peak at ~32 Mb, fine mapping identified a significantly associated region from 32,256,760 – 32,388,077 ($p=0.003$ – 0.009 , respectively). This region contained two known genes, Vitrin (VIT) and Striatin (STRN). Sequencing of the coding exons and splice sites of both genes did not identify any changes between affected and unaffected dogs. However, an 8 base pair deletion (32,373,916–32,373,923) in the 3' untranslated region (UTR) of the canine Striatin gene (Figure 2A,2B) was observed in 57 (16 homozygous, 41 heterozygous) of 61 ARVC-affected boxers but in only 9 (0 homozygous, 9 heterozygous) of 38 unaffected boxers, yielding a significant association signal ($p=0.005$). This sequence variant was not present in 100 unaffected dogs of eleven different breeds (non-boxers).

Markers from both the 32 and 36 Mb peaks were in reasonably strong linkage disequilibrium with each other (all pairwise $r^2 \geq 0.40$) despite the physical distance, but the deletion in the 3' UTR of STRN displayed much lower linkage disequilibrium with the haplotype containing the strongest markers (all pairwise $r^2 < 0.07$). Using a multimarker approach confirmed association of at least two haplotypes with the disease, one containing the three strongest markers at 36 Mb and another containing the deletion at 32 Mb. For example, a joint model containing both the deletion ($p=0.0006$) and a marker at chromosome 17: 32,194,234 ($p=0.0012$) demonstrated significant association beyond that of the markers at chromosome 17:36,233,479 to the disease. The same single marker logistic regression showed an odds ratio of 15.00 (95% CI: 3.87–58.08) overall for the deletion in this group of dogs. Pairwise models containing the deletion and each of the other three strongest markers showed similar joint significance (all $p < 0.05$). These data suggest an additional gene in or near the 36Mb region that may have a modifying impact on the disease.

Dogs that were homozygous for the deletion at the 3' UTR of STRN had more severe disease, based on ventricular premature complexes (VPC) number than heterozygous dogs ($p=0.001$) (Figure 3A). Homozygous dogs recorded 1,091–32,000 VPCs/24 hours (median of 5,102) and heterozygous ARVC dogs 109–19,000 VPCs/24 hours (median of 2,515) (Figure 3B). Normal adult healthy dogs have a median of 2 VPCs/24 hours (Meurs et al. 2001).

Secondary structure prediction of the 3'UTR determined an alteration in the stem loop formation in mutant allele as compared to the normal allele (Figure 4A, 4B). The miRBase database did not identify any miRNA binding sites within a 50,000 base pair region flanking the 3' UTR of STRN.

Expression analysis of mRNA levels in cardiac tissue identified a significant reduction ($p=0.04$) in Striatin between 2 non-Boxer controls and 4 ARVC dogs but not between heterozygous and homozygous mutant genotypes (Figure 5).

Western blot analysis also identified a significant reduction in Striatin protein between 2 non-Boxer controls and 4 ARVC dogs ARVC cases ($p=0.03$), but not between heterozygous and

homozygous mutant genotypes (Figure 6). Similar western blot results were obtained with both the striatin polyclonal and striatin monoclonal antibodies.

Striatin protein was localized within the myocardium by confocal microscopy. A polyclonal antibody to striatin, which is reported to not cross react with SG2NA, was used for immunolabeling. The most intense labeling occurred at the intercalated disk. This colocalized with labeling of other desmosomal proteins (plakophilin 2, plakoglobin and desmoplakin) in the normal dog (Figure 7). Some labeling of the nucleus and Z disc was also noted. Evaluation of homozygous-mutant ARVC-affected boxers demonstrated a varied appearance of Striatin when co-labeled to Plakophilin -2 in comparison to the normal dog (Figure 8).

Discussion

This genome-wide association study identified several candidate regions exhibiting association with the disease phenotype canine ARVC. The most significant region was found on canine chromosome 17. A novel sequence variant was identified on this chromosome in the highly associated region at 32,194,234 at the 3'UTR of the gene Striatin (STRN), which codes for a protein localized to the intercalated disc that co-labels to several desmosomal proteins. Boxer dogs homozygous for this variant were all affected, and on average were more severely affected than dogs heterozygous for the variant although there was overlap between the two groups. Thirty nine of 59 affected boxers were heterozygous for the variant, which is consistent with a co-dominant inheritance. Penetrance was 100% in homozygotes in this study, but only about 82% in heterozygous dogs.

Striatin is one of three proteins in a family that contain a coiled-coil structure, WD repeat domain, caveolin binding motif, and calcium dependent calmodulin binding site (Gaillard et al. 2001; Castets et al. 1996). Striatin has been previously described as a scaffolding protein functioning in a calcium dependent manner involved in both signaling and trafficking, and highly expressed in neurons (Gaillard et al. 2001; Gaillard et al. 2006). Although the role of Striatin in the heart has not been well described, expression in cardiac muscle has been reported (Yanai et al. 2005). In this study we have demonstrated that Striatin localizes to the cardiac intercalated disc and co-localizes to desmosomal proteins previously documented as being involved in the pathogenesis of human ARVC including Plakophilin-2, Desmoplakin and Plakoglobin. The facts that Striatin binds calmodulin in a calcium dependent manner and has a caveolin binding site are additional intriguing aspects of this protein that support its role in cardiac disease and may lend insight into the mechanisms of arrhythmias associated with this disease as well as the development of the unique myocardial fibrofatty infiltrate (Gaillard et al. 2006; Castets et al. 1996). To the best of our knowledge, Striatin has not previously been considered as a candidate for ARVC in humans.

Although Striatin has been demonstrated to be highly expressed in neurons, none of the dogs evaluated had obvious neurologic deficits (Gaillard et al. 2001; Gaillard et al. 2006). However, the identification of neurologic deficits would have been unexpected in these dogs given the results of previous animal studies. When expression of Striatin in the brain was reduced by 60% in rats, none of the rats demonstrated paralysis or any neurologic deficits in motor coordination. No overt behavioral changes in grooming, drinking and feeding were observed (Bartoli et al. 1999). Although nocturnal spontaneous locomotor activity was decreased by 30% compared to control rats, it is unlikely that owners of pet dogs would detect this level of nocturnal behavior change.

In this study, an eight base pair deletion was observed in the 3' UTR of the canine Striatin gene. Mutations in the 3' UTR of the transforming growth factor B3 gene have previously been associated with the development of ARVC in human beings as well (Beffagna et al. 2005).

The 3' UTR plays an important role in translation, localization and stability of mRNA and is rich in regulatory elements (Chatterjee et al. 2009; Chen et al. 2006). How mutations in the 3' UTR impact gene function and lead to the development of disease is not well understood (Chatterjee et al. 2009). Mutations at the 3' UTR may affect the termination codon, poly A signal, miRNA binding sites and/or secondary structure, and can cause translation deregulation (Chatterjee et al. 2009). The deletion identified in this study is not localized to the termination codon, poly A tail or a known canine miRNA binding site. However it does appear to alter the local secondary structure of this region. Since the secondary structure of the 3' UTR plays an important role in the interaction of mRNA with associated proteins, a change in the sequence that changes the secondary structure may alter its interaction with proteins or the stability of the mRNA which may contribute to the decreased expression of Striatin observed here (Chatterjee et al. 2009). The reduction in mRNA and protein observed in the ARVC dogs reported here could be associated with structural changes that prevent transcription or destabilizes the mRNA leading to degradation, thereby lowering the level of available mRNA.

A second highly associated region was identified with peak marker association at ~36 Mb (36,225,013; 36,233,479). The immediate surrounding region of this peak did not contain any defined genes and evaluation of two positional candidate cardiac genes, SLC8A1 and KCNG3, flanking this region did not identify any variations within their coding or splice site regions, or any alteration in their expression. However, the moderate linkage disequilibrium at further distances suggests that more intensive examination of a much broader region may be required to evaluate this area for additional genetic risk factors.

Four dogs originally diagnosed with ARVC did not have the deletion. There are a few possible explanations for this finding. One possibility is that these four dogs were incorrectly phenotyped. The diagnosis of ARVC can be quite challenging in both human beings and in the dog. Diagnosis is generally based on the presence of a combination of major and minor criteria including family history, global and regional functional and structural changes and electrocardiographic findings (Awad et al. 2008; Basso et al. 2009; Sen-Chowdhry et al. 2010). The dogs in this study were selected from a large pool of client owned canine patients in an ongoing study of canine ARVC. Although all of the dogs did have echocardiographic imaging and 24 hour ambulatory electrocardiograms performed, it was difficult to justify more invasive diagnostic testing including MRI for each dog unless it would be of medical benefit to the dogs in question. Therefore, although MRI would have provided additional supportive information on structure and function of the right ventricle for diagnosis of these dogs, it was only possible to assess a subset of the affected dogs. Likewise, it would have been valuable to have had a histopathologic diagnosis on all of the ARVC dogs but some owners declined a pathologic evaluation of their pet (Basso et al. 2004; Baumwart et al. 2009). None of the 4 dogs in question had MRI or histopathologic analysis performed. Therefore, it is possible that the dogs were incorrectly phenotyped as affected and actually had other disease processes that mimic ARVC. An additional possibility is that, as in human beings, ARVC may be a disease of significant genetic heterogeneity in the dog (Awad et al. 2008; Basso et al. 2009; Herren et al. 2009; van der Zwaag et al. 2009). In human beings, more than one hundred pathogenic variants have been identified in eight genes at this time (van der Zwaag et al. 2009). It is possible that the canine form of ARVC may be associated with more than one genetic mutation and likely more than one gene. Although it is true that the boxer dog is a pure breed dog with a fairly closed gene pool it has an estimated heterozygosity of approximately 47% (Irion et al. 2003). The development of more than one novel mutation is possible, especially given the significant genetic heterogeneity of the disease in human beings. Arrhythmogenic right ventricular cardiomyopathy in these four dogs (7% of the ARVC cases) may be due to a different, yet to be identified, genetic variant. In human beings, approximately 60 % of ARVC cases do not have a mutation in a known disease causing gene (Sen-Chowdhry et al. 2010).

Likewise, eleven of the thirty five dogs classified as controls were found to be heterozygous for the deletion. In this study we classified dogs as unaffected controls if they were at least 6 years of age and had less than 100 VPCs/24 hours. Boxer ARVC is an adult onset disease with a variable age of onset. The diagnosis of canine ARVC has been reported in dogs as young as 1 year of age and as old as 13 years of age with a median age of onset of 6 years of age (Harpster 1991). It is possible that dogs with the deletion who were not yet demonstrating the ARVC phenotype will express the disease at an older age. The variant described in this study had an approximately 72% penetrance. In human beings, ARVC is a disease with age related, relatively low penetrance and variable expressivity (Herren et al. 2009; Basso et al. 2009). In some kindreds with the autosomal dominant form of ARVC the penetrance may be as low as 20–30% (Sen-Chowdhry et al. 2005). Many individuals with a known genetic mutation will never develop clinically significant disease and even within the same family, some individuals will have a more benign disease course than others (Awad et al. 2008; Sen-Chowdhry et al. 2005). Degree of penetrance may be somewhat dependent on the specific causative gene. Desmoglein-2 mutations have a reported penetrance of 58–75% depending on the diagnostic criteria. Plakophilin-2 has a reported penetrance of 49% (Dalal et al. 2006). Penetrance in individuals with Plakophilin-2 mutations is both age and gender dependent with older and male individuals having a higher penetrance. Therefore, the expressivity of 72% observed in this study is consistent with that previously reported in human beings with ARVC. The incomplete penetrance and variability of expressivity in ARVC likely suggests the role of environmental factors and genetic modifiers in the presentation of this disease and supports further investigation of the second strongly associated region at 35–38.5 Mb (Awad et al. 2008).

Conclusion

In conclusion, we report here an 8 base pair deletion in the 3'UTR of a novel gene, Striatin (STRN), in this canine model of ARVC. Striatin is a protein localized to the intercalated disc that colocalizes with several desmosomal proteins and has both calcium dependent calmodulin and a caveolin binding sites. We suggest that Striatin may serve as a novel candidate gene for human ARVC. A second region of statistical significance may suggest that modifying factors are present and deserves further study.

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Figure 1. Genome-wide association mapping of canine ARVC. A 10 Mb region on canine chromosome 17 exhibited genome-wide significant association ($p_{\text{genome}} < 0.04$) (A). Close up of the chromosome 17 peaks (B).

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A **B** **Figure 3.**

Electrocardiogram from a boxer dog with canine ARVC (A). The black bar indicates a run of ventricular tachycardia that occurred after several normal sinus complexes. Dogs that were homozygous for the mutation had more severe disease based on ventricular premature complex number in comparison to dogs that were heterozygous for the mutation ($p=0.001$) (B).

Figure 4. Secondary structure prediction of the 3' untranslated region determined an alteration in both stem and loop formation in the ARVC dog in comparison to the control. (A, control dog; B, ARVC dog; arrow indicates region of deletion)

Figure 5.

Expression analysis of mRNA levels in cardiac tissue identified a significant reduction ($p=0.04$) in Striatin between 2 non-Boxer controls and 4 ARVC dogs. The length of each box indicates the range of values and the horizontal line indicates mean for the population.

Figure 6.

Representative image of protein analysis of Striatin in right ventricular myocardial sections. Lane 1 contains a non-boxer control; lane 2 contains a heterozygous ARVC dog; lane 3 contains a homozygous ARVC dog. Striatin protein as measured by corrected Optical Density was significantly reduced between ARVC cases ($n=4$) and controls ($n=2$) ($p=0.03$). Quantification of protein levels for 2 non-boxer controls and 2 heterozygous and 2 homozygous ARVC boxers is expressed at the bottom as a percentage of control.

Figure 7.

Striatin colocalizes with three desmosomal proteins. Confocal microscopy of normal beagle myocardium. Immunolabeling for striatin polyclonal antibody is green, red for desmosomal proteins (plakophilin 2, plakoglobin and desmoplakin) and the nuclei in the merged image are stained blue with DAPI. A no primary negative control is included for non-specific labeling. Bar=5μm.

Figure 8. Representative images of immunofluorescence studies demonstrating Striatin (green) and Plakophilin-2 (red) colocalizing in the desmosomal region of the cardiac myocyte in a control non-boxer dog (A), heterozygous ARVC dog (B) and homozygous ARVC dog (C).