Distinguishing Arrhythmogenic Right Ventricular Cardiomyopathy/Dysplasia–Associated Mutations From Background Genetic Noise

Jamie D. Kapplinger, BA,* Andrew P. Landstrom, BS,* Benjamin A. Salisbury, PhD,‡ Thomas E. Callis, PhD,‡ Guido D. Pollevick, PhD,‡ David J. Tester, BS,* Moniek G. P. J. Cox, MD,§ Zahir Bhuiyan, MD, PhD,¶ Hennie Bikker, PhD,¶ Ans C. P. Wiesfeld, MD, PhD,** Richard N. W. Hauer, MD,§ J. Peter van Tintelen, MD, PhD,†† Jan D. H. Jongbloed, PhD,‡ Hugh Calkins, MD,‡ Daniel P. Judge, MD,‡ Arthur A. M. Wilde, MD, PhD,‡# Michael J. Ackerman, MD, PhD*†

Rochester, Minnesota; New Haven, Connecticut; Utrecht, Amsterdam, and Groningen, the Netherlands; and Baltimore, Maryland

Objectives
The aims of this study were to determine the spectrum and prevalence of “background genetic noise” in the arrhythmogenic right ventricular cardiomyopathy/dysplasia (ARVC) genetic test and to determine genetic associations that can guide the interpretation of a positive test result.

Background
ARVC is a potentially lethal genetic cardiovascular disorder characterized by myocyte loss and fibrofatty tissue replacement of the right ventricle. Genetic variation among the ARVC susceptibility genes has not been systematically examined, and little is known about the background noise associated with the ARVC genetic test.

Methods
Using direct deoxyribonucleic acid sequencing, the coding exons/splice junctions of PKP2, DSP, DSG2, DSC2, and TMEM43 were genotyped for 93 probands diagnosed with ARVC from the Netherlands and 427 ostensibly healthy controls of various ethnicities. Eighty-two additional ARVC cases were obtained from published reports, and additional mutations were included from the ARVD/C Genetic Variants Database.

Results
The overall yield of mutations among ARVC cases was 58% versus 16% in controls. Radical mutations were hosted by 0.5% of control individuals versus 43% of ARVC cases, while 16% of controls hosted missense mutations versus a similar 21% of ARVC cases. Relative to controls, mutations in cases occurred more frequently in non-Caucasians, localized to the N-terminal regions of DSP and DSG2, and localized to highly conserved residues within PKP2 and DSG2.

Conclusions
This study is the first to comprehensively evaluate genetic variation in healthy controls for the ARVC susceptibility genes. Radical mutations are high-probability ARVC-associated mutations, whereas rare missense mutations should be interpreted in the context of race and ethnicity, mutation location, and sequence conservation. (J Am Coll Cardiol 2011;57:2317–27) © 2011 by the American College of Cardiology Foundation
Arrhythmogenic right ventricular cardiomyopathy/dysplasia (ARVC) is a heritable cardiac disease characterized by fibrofatty replacement of the right ventricle, with a prevalence of 1 in 1,000 to 5,000 individuals (1,2). It is a notable cause of sudden cardiac death (SCD) secondary to fatal ventricular arrhythmias, and sudden death is a common first manifestation of the disease (3). Despite its name, ARVC can involve the left ventricle, with a prevalence of 1 in 23,000, which is approximately 5% of the total ARVC cases (4). This variable clinical course may include: 1) a subclinical phase with concealed structural abnormalities, during which the affected patient may present with SCD as the sentinel manifestation of the disease; 2) an overt electrical disorder with palpitations and syncope due to tachyarrhythmias stemming from the right ventricle; and 3) severe right ventricular or biventricular failure requiring cardiac transplantation or resulting in death (5).

Roughly one-half of all ARVC is recognized as familial, with an autosomal dominant inheritance as well as incomplete penetrance and variable expressivity. To date, mutations in several ARVC susceptibility genes that encode essential desmosomal proteins have been identified: PKP2-encoding plakoglobin, DSP-encoding desmoplakin, DSG2-encoding desmoglein 2, DSC2-encoding desmocollin 2, JUP-encoding plakophilin, and TMEM43-encoding transmembrane protein 43 (4). Desmosomes, also known as macula adherens, are macromolecular cellular structures composed of plasma membrane proteins involved in linking intracellular intermediate filaments into a cell-spanning network, allowing effective transmission of force across cells. It is believed that functional deficits in these proteins imparted by genetic mutations result in cardiocyte detachment and cell death, while subsequent inflammation results in fibrofatty remodeling. In addition, it has been proposed that altered expression of ARVC susceptibility genes, specifically DSP, might lead to altered Wnt/b-catenin and Tcf/Leu signaling, causing the fibrofatty remodeling and SCD predisposition of ARVC (6).

Although nondesmosomal genes have been identified as rare causes of ARVC, the 3 desmosomal genes PKP2, DSP, and DSG2 harbor the majority of identified mutations, accounting for approximately 40% of ARVC cases in studies from the Netherlands (7,8), Italy (9), and the United States (10). In particular, PKP2 represents the most common gene mutated in ARVC, with a prevalence ranging from 40% (identified as the sole cause of ARVC in the cohort [3,7]) to approximately 10% (9). The variable phenotypic expression of this disease and the proclivity for inherited SCD risk make the identification of pathogenic disease biomarkers critical.

Recently, understanding of the genetic causes of ARVC has matured from the research bench to a clinically available commercial genetic test with an estimated yield of approximately 50% in patients with a likely diagnosis of ARVC (11). As with other clinical tests, a true understanding of the extent and spectrum of genetic variation found in a healthy population is necessary for correct interpretation. Although significant research has been dedicated to identifying pathogenic mutations, little is known about the background genetic variation in genes constituting the clinically available ARVC genetic tests. Understanding this signal-to-noise ratio is critical to determining whether an identified variant of undetermined or uncertain significance might be the biomarker responsible for disease in the proband or whether it is a rare genetic variant with no relevance to the disease state. To this end, we sought to determine the spectrum and prevalence of background genetic variation that would meet the criteria of a “mutation” on the ARVC genetic test and to use this information to guide interpretation of a positive genetic test result.

**Methods**

**Study cohorts.** From 1997 to 2007, patients with ARVC were referred to the Academic Medical Center in Amsterdam, the University Medical Center Groningen in Groningen, and the University Medical Center Utrecht in Utrecht, the Netherlands, for comprehensive ARVC genetic testing after receipt of written informed consent. For each proband, a history and physical examination, 12-lead electrocardiography, 24-h Holter monitoring, exercise testing, and 2-dimensional transthoracic echocardiography were conducted, as described previously (7,8).

To maximize the pre–genetic test possibility that a case-derived mutation is pathogenic, 93 “clinically definite,” unrelated cases were used for this study. ARVC diagnosis was based on the 2010 update (12) of the 1994 diagnostic criteria of the Task Force of the European Society of Cardiology/International Society and Federation of Cardiology (13). Similarly, 427 unrelated, ostensibly healthy subjects from various racial and ethnic backgrounds were subjected to ARVC genetic testing by Transgenomic Inc. (New Haven, Connecticut). These control individuals were volunteers recruited and genotyped by Transgenomic Inc. as part of the clinical ARVC genetic test validation process. At the time of genotyping, ethnicity, sex, and age at diagnosis or genotyping, as appropriate, were recorded for each case and control subject.
To expand the spectrum of ARVC case mutations, 82 ARVC index cases genotyped in a recent U.S. ARVD/C Registry (Baltimore, Maryland) study by den Haan et al. (10) were included, yielding a combined ARVC case cohort of 175 probands from the Netherlands and the United States. In addition, for analysis of linear topology and primary sequence species conservation, mutations from the ARVD/C Genetic Variants Database (14) (University Medical Center Groningen, Groningen, the Netherlands) were incorporated.

**Genetic analysis.** The genomic deoxyribonucleic acid (DNA) of all 427 controls and 93 Dutch cases was analyzed for mutations in the translated exons and the splice site regions of the major ARVC susceptibility genes: PKP2-encoding plakophilin 2, DSP-encoding desmoplakin, DSG2-encoding desmoglein 2, DSC2-encoding desmocollin 2, and TMEM43-encoding transmembrane protein 43. ARVC cases were additionally genotyped for JUP-encoding junction plakoglobin (also known as PKGB); however, because this gene was not genotyped for control subjects, it was excluded from statistical comparison. Among the 93 Dutch cases, identified mutations were absent in 400 reference alleles derived from unrelated, ostensibly healthy, ethnically matched Dutch volunteers. ARVC cases from the U.S. ARVD/C Registry were genotyped for all genes above with the exception of TMEM43, and all mutations were absent in 400 reference alleles derived from unrelated, ostensibly healthy, ethnically matched volunteers (Coriell Institute for Medical Research, Camden, New Jersey). Mutation analyses were performed using polymerase chain reaction followed by automated DNA sequencing.

**Genetic variant and mutation interpretation.** Genetic variants predicted to alter the coding protein, through missense alteration of a codon, alteration of the canonical splice site, both in-frame and frame-shift insertion or deletion mutations, or nonsense mutations resulting in a premature truncation, were identified for this study. Any such variant observed in only ARVC cases and absent in both the ethnically-matched controls as well as the 427-control cohort, or a variant uniquely identified in 1 control cohort subject, was annotated as a “mutation.” Importantly, the designation of “mutation” herein is not meant to imply pathogenicity or even functional relevance to the respective protein. Rather, these are variants that, had they been discovered during the course of a clinical ARVC genetic test, would be considered a possible pathogenic variant. Mutations were classified using standard nomenclature. In-frame and frame-shift insertions and deletions, splice junction, and nonsense mutations were designated as “radical” mutations. As with the designation of “mutation,” the designation of “radical” is not meant to imply disease pathogenicity but rather to serve as a descriptor for analysis of a class of mutations.

**Primary sequence and species conservation analysis.** Identified mutations were overlaid on the linear protein topology of each of the ARVC-associated gene products. The linear topology was annotated with known binding or functional domains,motifs, and predicted secondary structure regions from published reports (15,16) as well as the UniProtKB/Swiss-Prot databank (17). Sequence conservation analysis was similarly conducted using primary sequences from the University of California Santa Cruz Genome Browser (18). To calculate the degree of conservation of individual residues across species, primary sequences from 44 species, including primates, other placental mammals, and nonmammalian vertebrates, were aligned. Degree of nonidentity was determined by calculating the number of primary sequences harboring an amino acid not identical to the human residue at that location (substitution).

**Statistical analysis.** Statistical analysis used the Fisher exact test and 2-sided t tests, as appropriate, with a threshold of significance of p < 0.05. Variance was expressed as mean ± SD. Sequencing conservation scores were analyzed using Wilcoxon nonparametric 2-sample tests. Due to a lack of ARVC cases matched by race or ethnicity, statistical comparisons between ARVC cases and non–Caucasian controls were not conducted.

**Results**

**Study cohorts.** Among the patients referred to the Dutch university medical centers, 93 index cases met the criteria for a “definite” diagnosis of ARVC according to the 2010 ARVC task force criteria (12,13,19). This Dutch Caucasian cohort was 29% female, with an average age at diagnosis of 37 ± 14 years. These cases were combined with the 82 ARVC cases of North American Caucasian subjects from the U.S. ARVD/C Registry study cohort (10) (42% female, diagnosed at 34 ± 13 years) to yield a combined ARVC case cohort of 175 probands, 35% female, with an average age at diagnosis of 35 ± 14 years.

The control cohort consisted of 427 unrelated, ostensibly healthy subjects from various racial and ethnic backgrounds, including 103 Caucasian–American (24.1%), 124 Asian (29.0%), 110 African (25.8%), 42 Hispanic or Latino (9.8%), 4 American–Indian (0.94%), and 12 multiracial subjects (2.8%). There were 32 subjects (7.5%) from a variety of other racial and ethnic backgrounds. To be included in a specific racial or ethnic group, each subject must have reported 4 grandparents from the same group, while multiracial subjects had 1 or more grandparents of different racial or ethnic origin. This cohort was 50.1% female, with an average age at genotyping of 41 ± 15 years. The cohort demographics for this study are summarized in Table 1.

**Mutation spectrum and prevalence by cohort.** Comprehensive genetic interrogation of the control and ARVC case cohorts identified mutations in both cohorts. As demonstrated in Figure 1, among the ostensibly healthy, cosmopolitan control cohort, 69 of 427 subjects (16.2%) hosted genetic variants that met our criteria to be considered rare
“mutations,” creating what would have been annotated as a possible “positive” ARVC genetic test result had the subject been an ARVC case. Although not meant to imply pathogenicity, we used this label for the purposes of further analysis of background genetic variation. Among these control cohort mutations, 19.7% localized to PKP2, 32.9% in DSP, 21.1% in DSG2, 14.5% in DSC2, and 11.8% in TMEM43.

To test the hypothesis that the frequency of mutations found in control subjects reflected random genetic variability, we compared the frequency of mutations with the relative size of the coding deoxyribonucleic acid (cDNA) for each of the genes. The 4,439 nucleotides that constitute the cDNA of PKP2 represent approximately 15.7% of the total cDNA for the ARVC-associated genes and approximately 19.7% of the mutations identified in controls localized to PKP2. Likewise, the frequencies of control mutations in DSP, DSG2, DSC2, and TMEM43 were similar to their respective relative cDNA size (Table 2).

In contrast, ARVC cases had an overall yield of 58.3%, with 102 of 175 subjects hosting mutations absent in at least 400 reference alleles. Among the mutation-positive ARVC cases, 78.4% of the mutations localized to PKP2, 2.7% to DSP, 13.5% to DSG2, 3.6% to DSC2, 0.9% to JUP, and 0.9% to TMEM43. A summary of the genetic variants identified in the ARVC and control cohorts is listed in Online Tables 1 and 2.

As shown in Figure 2, while there was an approximately 3.5-fold higher genetic test yield for ARVC cases than controls (58.3% vs. 16.2%, p < 5 × 10^-24), approximately 1 in 6 healthy subjects genotyped met the criteria for a mutation and a positive ARVC genetic test result. This
so-called background genetic variation was largely missense mutations, as only 2 of 427 control subjects (0.47%) hosted radical mutations versus 75 of 175 ARVC cases (42.9%) \((p < 9.8 \times 10^{-44})\), while 68 of 427 controls (15.9%) hosted missense mutations versus 36 of 175 ARVC cases (20.6%) \((p = NS)\). The 2 radical mutations found in 2 control subjects were PKP2-IVS5-1G>A and DSP-S2843-R2846del. A small percent of control subjects hosted 2 or more mutations (9 of 427, 2.11%) compared with a higher percent of ARVC cases (8.00%) compared with either the Caucasian controls (0.97%, \(p < 8.1 \times 10^{-12}\)) or the non-Caucasian controls (0.31%). There was also a significantly higher percent of PKP2 missense mutations in ARVC cases (9.71%) than in Caucasian controls (0.97%, \(p < 0.005\)), while 4.01% of non-Caucasian controls hosted PKP2 missense mutations.

Whereas mutations localizing to PKP2 were more prevalent in ARVC cases than Caucasian controls, the prevalence of mutations localizing to DSP demonstrated no statistical difference between ARVC cases and Caucasian control subjects (6.17% vs. 1.71%, respectively) \((p > 0.05)\). The prevalence of mutations in healthy non-Caucasian control subjects was 6.48%. As shown in Figure 4C, a higher percent of ARVC cases hosted mutations in DSG2 compared to Caucasian controls (8.57% vs. 1.94%, \(p < 0.05\)), while there was no difference between the background genetic variation rate of non-Caucasians and ARVC cases. This difference was due to an overrepresentation of missense mutations among ARVC cases (8.00%) compared with Caucasians (1.94%, \(p < 0.05\)).

There was no discernible difference in the percent of patients with DSG2 (Fig. 5A) or TMEM43 (Fig. 5B) mutations between the ARVC and both control subcohorts, although the overall prevalence of mutations in these genes was low.

**Founder mutations.** Although the ARVC case cohort analyzed was composed of unrelated probands, we noted several mutations that were found in multiple ARVC cases that were absent in control subjects. To determine whether these potential founder mutations affected the overall yield of genetic testing among these ARVC cases, we identified and reanalyzed the cohort yield excluding these potential founder mutations: PKP2-IVS10-1G>C, which was identified in 13 ARVC cases (4 Dutch), PKP2-C796R mutation in 9 cases (all Dutch), and the PKP2-R79X nonsense mutation in 8 cases (6 Dutch). When each of these potential founder mutations was removed from the 2 ARVC cohorts, the overall yield of mutations in the ARVC cases dropped from 58.3% to 49.7% (63.4% to 43.0%) in the Dutch ARVC cohort and 52.4% to 39.0% in the North American ARVC cohort.

**Mutation spectrum and prevalence by gene.** To determine whether the high background rate of genetic variation might be gene specific, as well as race or ethnicity specific, we next calculated the percent of mutation-positive subjects for each gene within the control cohort, both Caucasian and non-Caucasian subsets, and the ARVC cohort (Figs. 4 and 5). As demonstrated in Figure 4A, there was a higher percent of subjects in the ARVC cohort (49.7%) with mutations in PKP2 than in Caucasian controls (0.97%, \(p < 2.7 \times 10^{-21}\)). While 4.32% of non-Caucasian controls hosted mutations in PKP2, statistical comparison with the ARVC cases could not be conducted, because of a lack of non-Caucasian ARVC cases available in this study. This difference in mutation frequency largely reflects a higher percent of radical mutations in the ARVC cases (42.3%) than among either the Caucasian controls (0% vs. \(8.1 \times 10^{-12}\)) or the non-Caucasian controls (0.31%). There was also a significantly higher percent of PKP2 missense mutations in ARVC cases (9.71%) than in Caucasian controls (0.97%, \(p < 0.005\)) while 4.01% of non-Caucasian controls hosted PKP2 missense mutations.

Bar graph of the number and percent of the arrhythmogenic right ventricular cardiomyopathy/dysplasia (ARVC) (blue background) and control (white background) subjects hosting radical (no lines) or missense (diagonal lines) mutations. Mutations identified in controls were subdivided into Caucasian and non-Caucasian ostensibly healthy individuals. Numbers within bars denote number of subjects positive for the respective mutation type in each cohort and cohort subset.

**Figure 3**

Prevalence of Mutations in Caucasian and Non-Caucasian Subjects

<table>
<thead>
<tr>
<th>Mutation Type</th>
<th>ARVC</th>
<th>Overall</th>
<th>Caucasian</th>
<th>Non-Caucasian</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radical</td>
<td>42.0%</td>
<td>20.6%</td>
<td>0.47%</td>
<td>0%</td>
</tr>
<tr>
<td>Missense</td>
<td>40.1%</td>
<td>36%</td>
<td>15.9%</td>
<td>5.3%</td>
</tr>
</tbody>
</table>

**Mutation spectrum and prevalence by race and ethnicity.** To determine whether there were race-specific or ethnicity-specific differences in mutation type or prevalence, particularly among missense mutations, we determined the frequency of mutations among Caucasian and non-Caucasian control subpopulations. Although the yield of mutations in control subjects was 16.2% overall, mutations in non-Caucasian subjects occurred at a higher frequency than in Caucasian control subjects (19.44% vs. 5.83%, respectively, \(p < 0.003\)). Specifically, as shown in Figure 3, ostensibly healthy Caucasian subjects had a much lower rate of missense mutations (6 of 103, 5.83%) than non-Caucasian control subjects (63 of 324, 19.4%; \(p < 0.0007\)). This was also lower than the overall prevalence of missense mutations among the Dutch and North American Caucasian ARVC cases (36 of 175, 20.6%; \(p < 0.0009\)). The prevalence of radical mutations across Caucasian and non-Caucasian subsets of the control cohort was extremely small (0% and 0.62%, respectively).

**Founder mutations.** Although the ARVC case cohort analyzed was composed of unrelated probands, we noted several mutations that were found in multiple ARVC cases that were absent in control subjects. To determine whether these potential founder mutations affected the overall yield of genetic testing among these ARVC cases, we identified and reanalyzed the cohort yield excluding these potential founder mutations: PKP2-IVS10-1G>C, which was identified in 13 ARVC cases (4 Dutch), PKP2-C796R mutation in 9 cases (all Dutch), and the PKP2-R79X nonsense mutation in 8 cases (6 Dutch). When each of these potential founder mutations was removed from the 2 ARVC cohorts, the overall yield of mutations in the ARVC cases dropped from 58.3% to 49.7% (63.4% to 43.0%) in the Dutch ARVC cohort and 52.4% to 39.0% in the North American ARVC cohort.
As each of these potential founder mutations localized to *PKP2*, exclusion of these mutations reduced the *PKP2*-specific yield from 49.7% to 32.6% (53.8% to 33.3% in the Dutch ARVC cohort and 45.1% to 31.7% in the North American ARVC cohort) while not altering other ARVC gene frequencies.

Mutation “hot spots” in desmoplakin and desmoglein 2. On the basis of the identification of key binding and functional

**Figure 4** Prevalence of Mutations in *PKP2*, *DSP*, and *DSG2*

Bar graph depicting the number and percent of the Caucasian control (white), non-Caucasian control (white with cross-hatching), and arrhythmogenic right ventricular cardiomyopathy/dysplasia (ARVC) case (blue) cohorts hosting mutations in *PKP2* (A), *DSP* (B), and *DSG2* (C). The overall yield of the genetic test (positive) was divided into radical mutations, missense mutations, and subjects hosting more than 1 mutation (multiple). Numbers within bars denote number of subjects positive for a mutation in the respective gene in each cohort. *p < 0.05; **p < 0.01; ***p < 0.0001.

**Figure 5** Prevalence of Mutations in *DSC2* and *TMEM43*

Bar graph depicting the number and percent of the Caucasian control (white), non-Caucasian control (white with cross-hatching), and arrhythmogenic right ventricular cardiomyopathy/dysplasia (ARVC) case (blue) cohorts hosting mutations in *DSC2* (A) and *TMEM43* (B). The overall yield of the genetic test (positive) was divided into radical mutations, missense mutations, and subjects hosting more than 1 mutation (multiple). Numbers within bars denote number of subjects positive for a mutation in the respective gene in each cohort. *TMEM43* was not genotyped in the U.S. ARVD/C Registry component of the ARVC case cohort (n = 93).
domains on the proteins constituting the desmosome (15,16), we next investigated whether missense mutations identified in ARVC cases might localize preferentially to critical protein domains compared to control mutations. As depicted in Figure 6, we identified regions on the linear topology of DSP and DSG2 that were apparent mutation “hot spots” in which a significantly larger number of missense case mutations were identified compared with controls. Specifically, an amino-terminal region of DSP encompassing 3 of the predicted alpha-helical bundles Z,
Table 3  Sequence Conservation and Missense Mutations in ARVC Genes

<table>
<thead>
<tr>
<th>Cohort</th>
<th>n</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>&gt;5</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>133</td>
<td>11</td>
<td>19</td>
<td>8</td>
<td>10</td>
<td>9</td>
<td>15</td>
<td>61</td>
<td>6.71</td>
</tr>
<tr>
<td>ARVC</td>
<td>81</td>
<td>35</td>
<td>11</td>
<td>6</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>17</td>
<td>2.85</td>
</tr>
</tbody>
</table>

p < 0.0001 (z-score = −5.61).

ARVC = arrhythmogenic right ventricular cardiomyopathy/dysplasia; mean = mean number of species that have a different residue at the location of the given variant for the cohort; n = total number of mutations localizing to the 5 ARVC-associated genes analyzed.

Table 4  Sequence Conservation and Missense Mutations in PKP2

<table>
<thead>
<tr>
<th>Cohort</th>
<th>n</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>&gt;5</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>7</td>
<td>5.74</td>
</tr>
<tr>
<td>ARVC</td>
<td>23</td>
<td>11</td>
<td>5</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>1.83</td>
</tr>
</tbody>
</table>

p < 0.0001 (z-score = −4.33).
n = total number of mutations localizing to PKP2; other abbreviations as in Table 3.

Y, and X (residues 250 through 604) contained 8 of 17 (47%) of the DSP missense mutations found in ARVC cases compared with 1 of 28 (3.6%) of the DSP missense mutations found in controls (p < 0.0008) (Fig. 6A). Likewise, the amino-terminal propeptide and cadherin repeats I to III domains (residues 24 through 388) of DSG2 demonstrated an overrepresentation of missense mutations compared with controls (16 of 21 vs. 2 of 20, p < 2.5 × 10⁻⁵) (Fig. 6B). These mutation hot spots localize to key regions of the desmosomal macromolecular complex, as demonstrated in Figure 7. Specifically, ARVC case mutations are more prevalent in a region of DSP and DSG2 that include, or are closely proximal to, respective protein binding domains critical to formation of the desmosome.

Mutations and primary sequence conservation in plakophilin 2 and desmoglein 2. In an attempt to more clearly define differences in ARVC case and healthy control missense mutations, we explored possible associations between the location of missense mutations and the conservation of the primary sequence across species for each of the ARVC susceptibility gene products. As demonstrated in Table 3, across the 5 ARVC-associated genes analyzed, mutations identified in control subjects were more likely to be localized to residues that were more highly substituted across species (mean 6.71 substitutions) than ARVC cases (mean 2.85 substitutions, p < 0.0001, z-score = −5.61).

Specifically, ARVC case-based missense mutations in PKP2 and DSG2 altered residues that were significantly more conserved across species than those missense mutations derived from controls. As shown in Table 4, among the 19 PKP2 missense mutations identified in control subjects, 13 localized to residues that were substituted in 3 or more species queried (mean 4.13). In contrast, among the 23 ARVC case mutations, 11 mutations localized to residues that were not substituted for other residues across species, yielding a mean degree of nonidentity of 1.65 (p < 0.0001, z-score = −4.33). Similarly, 18 of 33 control missense mutations in DSG2 localized to residues with substitutions in more than 5 species (mean 9.15), while 15 of 27 ARVC mutations localized to nonsubstituted residues (mean 1.96, p < 0.0001, z-score = −4.66) (Table 5). For DSP, DSC2, and TMEM43, we found no relationship, or we were underpowered to determine an association, between the degree of species conservation and whether a residue was altered in an ARVC case or control subject.

Discussion

ARVC genetic test interpretation. Understanding the background rate of genetic variability is critical to interpreting the results of a genetic test. Recently, the spectrum of background genetic variation in the genes encoding proteins implicated in the pathogenesis of another potentially lethal arrhythmia syndrome, long-QT syndrome, has been elucidated (20). For long-QT syndrome, the background mutation rate for its 3 canonical genes, KCNQ1, KCNH2, and SCN5A, was about 5%. Here, we report an initial background mutation rate, unadjusted for ancestry, in the analyzed ARVC susceptibility genes to be approximately 16%, or 3-fold higher than the genetic noise prevalence seen in long-QT syndrome. This poses several challenges to interpreting the ARVC genetic test, as in 6 healthy individuals would meet current criteria for a so-called positive ARVC genetic test result, even with proper qualification of these rare “mutations” with the clinically ambiguous designation as a “variant of uncertain significance.” Herein, we hope to provide some initial genetic associations to guide physicians in determining an estimated probability of pathogenicity following the identification of a rare variant in an ARVC susceptibility gene.

Given the background rate of mutations in the control cohort compared with ARVC cases as well as the demon-
strated differences in this signal-to-noise ratio from mutation type (radical vs. missense) and race and ethnicity, we created a summary of factors we believe alter the likelihood that a given variant is pathogenic (Fig. 8). First, we observe that radical mutations (insertions or deletions, splice junction mutations, and nonsense mutations) are significantly more prevalent in ARVC cases compared with controls (49.9% vs. 0.47%, respectively, p < 9.8 × 10⁻⁴⁴), so an ARVC genetic test result indicating the presence of this type of genetic variant has a high likelihood of being associated with ARVC pathogenicity. Notably, such radical mutations constituted the majority (75 of 102, 73.5%) of genetic alterations identified in mutation-positive ARVC cases.

If the ARVC genetic test indicates a missense mutation, we identified 3 associations that might strengthen or weaken the possibility of ARVC pathogenicity for the variant in question. On the basis of our observation that ostensibly healthy Caucasian subjects had a significantly lower rate of missense mutations than ARVC cases (5.83% vs. 20.6%, respectively, p < 0.0009), a rare missense mutation identified in a Caucasian patient is more likely pathogenic than one identified in a non-Caucasian patient. Furthermore, specific amino-terminal regions of DSP and DSG2 may contain mutation “hot spots,” as each was found to host significantly more missense mutations in ARVC cases than in controls (p < 0.0008 and p < 2.5 × 10⁻⁵, respectively). Finally, there were significantly more ARVC missense mutations involving residues within PKP2 and DSG2 that were highly conserved across species. Given the observation that missense mutations in ARVC cases demonstrated a rate of substitution of 1.65 and 1.19 for PKP2 and DSG2, respectively, compared with 4.13 (p < 0.0001) and 9.95 (p < 0.0001) for control subjects, respectively, the identification of a missense mutation that involves a highly conserved residue is more likely pathogenic.

When properly interpreted, ARVC genetic testing ultimately affords the possibility of early detection of preclinical disease, identification of at-risk family members, and genetic counseling for this sudden death–predisposing disease. However, because of the potential clinical impact of such a test, and multiple factors that might influence how and when a genetic abnormality might manifest as disease, ARVC genetic testing should not be viewed as a perfectly binary (yes or no, positive or negative) test. The presence of a genetic mutation alone cannot override clinical judgment regarding the diagnosis of ARVC, nor should the absence of a mutation in the setting of compelling clinical evidence call the diagnosis into question. Regardless of the strength of statistical assessment that a mutation is pathogenic, genetic test results should be viewed as probabilistic and as 1 component of an overall clinical assessment.

**Background genetic noise and occult disease.** Although it is possible that occult, undiagnosed ARVC is present in a small number of the ostensibly healthy volunteers genotyped in the control cohort, it is highly unlikely that this accounts for the significant genetic variability found in our control population. Although most estimates place the prevalence of ARVC at 1 in 5,000 among the general population (2), a conservative estimate of 1 in 1,000 would suggest a 50% chance that only a single subject within the 427-control cohort has occult ARVC (1). Even if the frequency of clinically silent ARVC in our cohort were 10-fold higher, this conservative estimate would constitute approximately 1% of the entire control cohort, ultimately resulting in a decrease of genetic noise from a 16% to 15% prevalence. In addition, the relative gene–specific frequency of mutations in ostensibly healthy subjects supports the hypothesis that these variants are randomly occurring background genetic variation. Although the majority (78%) of ARVC case mutations localized to PKP2, control mutations were dis-

---

**Table 5** Sequence Conservation and Missense Mutations in DSG2

<table>
<thead>
<tr>
<th>Cohort</th>
<th>n</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>&gt;5</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>33</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>18</td>
<td>9.15</td>
</tr>
<tr>
<td>ARVC</td>
<td>27</td>
<td>15</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>1.96</td>
</tr>
</tbody>
</table>

*p < 0.0001 (z-score = -4.66),

n = total number of mutations localizing to DSG2; other abbreviations as in Table 3.

---

**Figure 8 Assessing Likelihood of Pathogenicity of a Positive ARVC Genetic Test Result**

Flow chart of factors influencing likelihood that a given arrhythmogenic right ventricular cardiomyopathy/dysplasia (ARVC) genetic test–identified mutation is pathogenic on the basis of comparison of mutations identified in ostensibly healthy controls versus definite ARVC cases. VUS = variant of undetermined or uncertain significance.
tributed among the ARVC genes in a manner reflective of the relative size of the gene.

It is also possible that the apparently high rate of mutations in healthy subjects might be somewhat inflated for this initial control cohort relative to future studies. One would expect that genetic interrogation of additional control subjects would re-encounter some of these unique genetic variants, while fewer new variants would be discovered. As per our criteria for mutation designation, a variant identified in more than 1 healthy subject was not considered a mutation, so genotyping additional control subjects could meaningfully reduce the estimated background mutation rate. One reason the apparent background rate is lower in the Caucasian subset of controls may be that, relative to other ancestries, more Caucasian controls have been interrogated genetically previously.

**Compound and digenic heterozygosity.** A study by Xu et al. (21) genetically interrogated 198 U.S. and Italian ARVC cases and identified mutations localizing to PKP2 in roughly 19% of the probands, with 42% of these PKP2-positive subjects, or 8% of the cohort overall, hosting at least 1 additional mutation in 1 of the ARVC susceptibility genes. This yield of PKP2 mutations is similar to a Danish ARVC cohort that demonstrated 8 of 53 subjects (15%) with PKP2 mutations (22). Furthermore, Xu et al. (21) presented a number of pedigrees of kindred hosting 2 ARVC mutations in trans, wherein coinherence of both mutations results in a more severe clinical phenotype compared to patients hosting only 1. In their analysis, compound and digenic heterozygosity accounted for the variable penetrance observed in their pedigrees and led the investigators to conclude that a single PKP2 mutation may be insufficient to potentiate clinical disease.

Although we observed a similar prevalence of multiple mutations in ARVC cases overall (7%), this was in the context of a higher percent of PKP2-positive subjects (49.7%, or 39.9% when excluding 3 potential founder mutations). Thus, among the subset of genotype-positive ARVC cases seen in this study, 93% had a single ARVC-associated mutation. This is similar to a recent study by Fressart et al. (23), which identified multiple mutations in 4% of ARVC cases. Furthermore, we identified 9 subjects (2%) in our ostensibly healthy control cohort who similarly hosted multiple mutations. Interestingly, a study by Bhuiyan et al. (8) recently found that mutations in DSC2 and DSG2, although less prevalent than mutations in PKP2, also occurred in the setting of another mutation in an ARVC-associated gene, perhaps indicating that compound and digenic heterozygosity might play a role outside of just PKP2 mutations.

Although compound and digenic heterozygosity clearly play a large role in explaining some of the incomplete penetrance and variable expressivity of the disease in ARVC families, we believe that the type and location of the mutation might be similarly important. We found the frequency of radical mutations in our control cohort to be 0.5%, in comparison with the ARVC case frequency of 43%. This difference alone provides significant statistical evidence that a radical mutation reported on an ARVC genetic test is likely pathogenic. Another possibility is that compound and digenic heterozygosity may play a modulatory role that is most salient in families with variable ARVC expression and may not play as meaningful a role in dictating disease penetrance in our cohorts.

**Study limitations.** Although this study is the first and largest systematic examination of the spectrum and prevalence of ARVC-associated background genetic variation, small sample sizes of both the ARVC case and control cohorts still represent a limitation, especially considering individual ethnogeographic strata. The observed statistical associations were based on a relatively small sample size compared with other similar studies in channelopathic disease and might be susceptible to sway by addition of a few outlier subjects and founder mutation (20). Despite this, the observed statistical associations appear robust, were based on the largest set of controls and one of the largest case collections examined to date, and are testable in future replication studies. Future studies incorporating additional phenotypically robust ARVC cohorts and thoroughly examined ostensibly healthy subjects genotyped for the equivalent of the ARVC genetic test panel will further validate and extend the generalizability of these observations.

As previously discussed, normal results on 12-lead electrocardiography, Holter monitoring, and echocardiographic analysis were not a prerequisite for inclusion in the control cohort. Thus, it is possible that a small number of the 69 “mutation-positive” controls may have had clinically silent ARVC, and a mutation identified in those subjects could be falsely considered part of the background genetic variation. As mentioned earlier, statistically, there should be only 1, at most 2, of the 427 control subjects who nevertheless had ARVC. Among these 69 mutation-positive controls, 2 are indeed suspicious for ARVC pathogenicity: a subject hosting the splice junction IVS5-1G>A mutation and one hosting the in-frame DSP-S2843-R2846del and a concomitant PKP2-V587I nonsense mutation. Although we acknowledge the possibility that some mutations among control subjects may represent incompletely penetrant mutations that may be pathogenic in another patient, for all variants to be pathogenic, the average penetrance of these variants must be extremely low, as the most conservative estimate of ARVC in the healthy population is 1 in 1,000. Notably, we cannot exclude the possibility that ARVC cases, particularly those hosting only 1 ARVC-associated mutation, do not carry a compound mutation in a yet unknown ARVC susceptibility gene.

**Conclusions**

In the first comprehensive study of genetic variation in ostensibly healthy subjects for ARVC susceptibility genes, we demonstrate that radical mutations are high-probability
ARVC-associated mutations, whereas rare missense mutations should be interpreted with great caution. A compilation of factors including mutation localization, the conservation of the primary sequence across species, and the race and ethnicity of the patient influence the probability of missense mutations truly being the pathogenic biomarker of disease.

Reprint requests and correspondence: Dr. Michael J. Ackerman, Mayo Clinic, Windland Smith Rice Sudden Death Genomics Laboratory, Guggenheim 501, Rochester, Minnesota 55905. E-mail: ackerman.michael@mayo.edu.

REFERENCES


Key Words: arrhythmogenic right ventricular cardiomyopathy ■ arrhythmogenic right ventricular dysplasia ■ diagnosis ■ genetic testing ■ mutation.

APPENDIX

For supplemental Tables 1 and 2, please see the online version of this article.