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Compound and Digenic Heterozygosity Contributes to Arrhythmogenic Right Ventricular Cardiomyopathy

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Objectives The aim of this study was to define the genetic basis of arrhythmogenic right ventricular cardiomyopathy (ARVC).

Background Arrhythmogenic right ventricular cardiomyopathy, characterized by right ventricular fibrofatty replacement and arrhythmias, causes sudden death. Autosomal dominant inheritance, reduced penetrance, and 7 desmosome-encoding causative genes are known. The basis of low penetrance is poorly understood.

Methods Arrhythmogenic right ventricular cardiomyopathy probands and family members were enrolled, blood was obtained, lymphoblastoid cell lines were immortalized, deoxyribonucleic acid was extracted, polymerase chain reaction (PCR) amplification of desmosome-encoding genes was performed, PCR products were sequenced, and diseased tissue samples were studied for intercellular junction protein distribution with confocal immunofluorescence microscopy and antibodies against key proteins.

Results We identified 21 variants in *plakophilin-2* (*PKP2*) in 38 of 198 probands (19%), including missense, nonsense, splice site, and deletion/insertion mutations. Pedigrees showed wide intra-familial variability (severe early-onset disease to asymptomatic individuals). In 9 of 38 probands, *PKP2* variants were identified that were encoded in *trans* (compound heterozygosity). The 38 probands hosting *PKP2* variants were screened for other desmosomal genes mutations; second variants (digenic heterozygosity) were identified in 16 of 38 subjects with *PKP2* variants (42%), including *desmoplakin* (*DSP*) (n = 6), *desmoglein-2* (*DSG2*) (n = 5), *plakophilin-4* (*PKP4*) (n = 1), and *desmocollin-2* (*DSC2*) (n = 1). Heterozygous mutations in non-*PKP 2* desmosomal genes occurred in 14 of 198 subjects (7%), including *DSP* (n = 4), *DSG2* (n = 5), *DSC2* (n = 3), and *junctional plakoglobin* (*JUP*) (n = 2). All variants occurred in conserved regions; none was identified in 700 ethnic-matched control subjects. Immunohistochemical analysis demonstrated abnormalities of protein architecture.

Conclusions These data suggest that the genetic basis of ARVC includes reduced penetrance with compound and digenic heterozygosity. Disturbed junctional cytoarchitecture in subjects with desmosomal mutations confirms that ARVC is a disease of the desmosome and cell junction. (J Am Coll Cardiol 2010;55:587–97) © 2010 by the American College of Cardiology Foundation

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Abbreviations and Acronyms

ARVC = arrhythmogenic right ventricular cardiomyopathy
DNA = deoxyribonucleic acid
DSC = desmocollin
DSG = desmoglein
DSP = desmoplakin
LV = left ventricle/ventricular
MRI = magnetic resonance imaging
PCR = polymerase chain reaction
PKP = plakophilin
RV = right ventricle/ventricular

Arrhythmogenic right ventricular cardiomyopathy/dysplasia (ARVC) has been defined as a primary right ventricular (RV) cardiomyopathy initially described by Marcus et al. (1) in the early 1980s. It is characterized by fibrofatty infiltration of the RV myocardium and clinically presents with ventricular arrhythmias, heart failure, syncope, and sudden death (1,2). The left ventricle (LV) might also be affected (3). In familial cases of ARVC, autosomal dominant inheritance with reduced penetrance has been reported and is believed to account for approximately 30% of cases (4). In the remaining sporadic cases, the etiology might be an acquired cause such as myocarditis (5,6) or an unidentified inherited disorder. To date, multiple genetic loci and 7 genes—including *desmoplakin* (*DSP*) (7), *plakophilin-2* (*PKP2*) (8), *desmoglein-2* (*DSG2*) (9), *desmocollin-2* (*DSC2*) (10), *transforming growth factor β3* (*TGF β3*) (11), *ryanodine receptor 2* (*RYR2*) (12), and *transmembrane protein-43* (*TMEM43*) (13)—have been identified in ARVC patients. In addition, 2 complex cardiocutaneous disorders with autosomal recessive inheritance in which cardiomyopathy is associated with woolly hair and palmoplantar keratoderma have been reported. These include Naxos disease with ARVC (14) and Carvajal syndrome associated with an LV cardiomyopathy (15). The genes identified for these disorders include homozygous mutations in *junctional plakoglobin* (*JUP*) in Naxos disease (16) as well as homozygous mutations in *DSP* in Carvajal syndrome (17). The most common gene variant identified in ARVC is *PKP2*, initially reported by Gerull et al. (8) to be mutated in approximately 25% of patients with autosomal dominant inherited disease. In the analysis of *PKP2*, an essential armadillo-repeat protein of the cardiac desmosome, they identified heterozygous mutations in 32 of 120 unrelated individuals with ARVC (8). Other investigations have confirmed these findings (18–21). Of genes identified to date causing ARVC, a majority encode desmosomal proteins. Within cardiomyocytes, 2 types of cell adhesion junctions are responsible for intercellular adhesion: the desmosomes and fascia adherens junctions (22). These cell adhesion junctions are both located at the cardiomyocyte intercalated disk, and both contain intracellular proteins that link the cytoplasmic domains of cadherins to components of the cytoskeleton. In cardiomyocytes, a variety of proteins interact to form functional cell–cell junctions. The DSGs and DSCs are connected to the desmin cytoskeleton by DSP, JUP (γ-catenin), and PKP2. These latter proteins, JUP and PKP2, are members of the armadillo family of nuclear and junctional proteins (23,24). The PKP-2 interacts with DSP, DSG, and intermediate filament proteins at sites within its N-terminus. The DSP and PKP2 are located only in desmosomes, whereas JUP participates as a linker in both desmosomes and adherens junctions. The adherens junctions are located at the ends of sarcomeres and are linked to sarcomeric actin through intracellular linker proteins, most notably members of the catenin family, including JUP, β-catenin, α-catenin, and p120 catenin.

In this study, we analyzed probands and family members for ARVC with a standardized clinical protocol either developed as part of the North American ARVD Registry (25) or with the standard Task Force criteria (Table 1) (26). All individuals were screened for mutations in all of genes encoding proteins involved in desmosomal function, even if a variant were already identified in any of these desmosome-encoding genes. We report identification of multiple mutations in these genes, including autosomal dominant heterozygous mutations in 26% of subjects (52 of 198), including 38 in *PKP2* and 14 in other desmosome-encoding genes.

Additionally, compound heterozygous mutations and digenic mutations were identified in 42% of the subjects (16 of 38) in whom *PKP2* mutations were identified. In addition, we demonstrate that many *PKP2* mutations have low penetrance and in many cases might not be the primary cause of the disease, contradicting the previously reported contention that *PKP2* is the major ARVC-causing gene, accounting for the cause of disease in 25% of ARVC patients.

Methods

Patient evaluation. After informed consent, probands were evaluated by noninvasive and invasive studies, including physical examination and history/family history, chest radiography, 12-lead electrocardiogram, echocardiography, and cardiac magnetic resonance imaging (MRI).

In most cases, the clinical evaluation followed the protocol of the National Institutes of Health–funded North American ARVD Registry (25), which included invasive studies including cardiac catheterization, ventricular angiography, and endomyocardial biopsy. In these subjects, all studies (noninvasive and invasive testing) were analyzed by core laboratories. Family members were evaluated with the noninvasive studies only (electrocardiogram, cardiac MRI, echocardiogram, chest X-ray, and physical examination

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Table 1 Task Force Diagnostic Criteria for ARVC

I. Global and/or regional dysfunction and structural alterations
Major
Severe dilation and reduction of RV ejection fraction with no (or only mild) LV impairment
Localized RV aneurysms (akinetic or dyskinetic areas with diastolic bulging)
Severe segmental dilation of the RV
Minor
Mild global RV dilation and/or ejection fraction reduction with normal LV
Mild segmental dilation of the RV
Regional RV hypokinesia
II. Tissue characterization of wall
Major
Fibrofatty replacement of myocardium on endomyocardial biopsy
III. Repolarization abnormalities
Minor
Inverted T waves in right precordial leads (V_2 and V_3) in people age >12 yrs, in absence of right bundle-branch block
IV. Depolarization/conduction abnormalities
Major
Epsilon waves or localized prolongation (>110 ms) of the QRS complex in right precordial leads (V_1–V_3)
Minor
Late potentials (signal-averaged ECG)
V. Arrhythmias
Minor
Left bundle-branch block type ventricular tachycardia (sustained and nonsustained) by ECG, Holter, or exercise testing
Frequent ventricular extrasystoles (>1,000/24 h) (Holter)
VI. Family history
Major
Familial disease confirmed at necropsy or surgery
Minor
Family history of premature sudden death (<35 yrs) due to suspected RV dysplasia

Familial history (clinical diagnosis based on present criteria). Data adapted from McKenna et al. (26). The diagnosis of arrhythmogenic right ventricular cardiomyopathy (ARVC) is fulfilled by the presence of 2 major, 1 major plus 2 minor, or 4 minor criteria from I to VI (25,26).
ECG = electrocardiogram; LV = left ventricle/ventricular; RV = right ventricle/ventricular.

with history/family history). In the subjects in whom genetic studies were performed but who declined enrollment in the Registry or international subjects not eligible to enroll in the Registry, the Task Force diagnostic criteria (Table 1) were used (26). Diagnostic criteria previously described by McKenna et al. (26) were used to determine affectation status. After informed consent, blood for deoxyribonucleic acid (DNA) extraction and lymphoblastoid cell line immortalization was obtained, as approved by the Baylor College of Medicine Institutional Review Board.

DNA sequencing analysis. Genomic DNA samples of the 143 U.S. and 55 Italian ARVC index cases (n = 198) were obtained from blood samples and immortalized lymphoblastoid cell lines as previously described (27) and amplified by polymerase chain reaction (PCR) with primers designed to amplify the coding exons of desmosome-encoding genes *PKP2, DSP, JUP, DSC2, DSG2,* and *plakophilin-4* (*PKP4*), and the intermediate filament-encoding gene *desmin* (*DES*). Other nondesmosomal genes were excluded from this analysis. The PCR products from the U.S. cohort were sequenced with Big Dye Terminator version 3.1 chemistry (Applied Biosystems, Foster City, California) and analyzed with an ABI 3730 DNA sequencer (Applied Biosystems). In addition, the 55 Italian ARVC index cases were screened for mutations by denaturing high-performance liquid chromatography and direct sequencing. Denaturing high-performance liquid chromatography analysis was performed with the use of WAVE Nucleic Acid Fragment Analysis System 3500HT with DNASep HT cartridge (Transgenomic, Ltd., Omaha, Nebraska). Temperatures for sample analysis were selected with the use of WaveMaker software version 4.0 (San Francisco, California). In all 198 subjects, therefore, we analyzed the entire coding sequence and the surrounding intronic sequences of DES and of the desmosomal genes including *PKP2*, *DSP*, *DSC2*, *DSG2*, *JUP*, *DSC2*, *DSG2*, and *PKP4*.

Cloning and sequencing of PKP2 complementary DNA. Total ribonucleic acid was isolated from lymphoblastoid cell lines with an RNeasy Mini Kit (Qiagen, Stanford, California) and subjected to random hexamer-primed complementary DNA synthesis with Superscript II (Invitrogen, Carlsbad, California). The *PKP2* complementary DNA was amplified by PCR with oligonucleotides specific for the complementary DNA sequence of *PKP2* (5′-CCAGCTGAGTACGGCTACATC-3′; 5′-TCAGTCTTTAAGGGAGTGGT-3′), cloned into TA-vectors (Topo TA Cloning Kit, Invitrogen) and then introduced into TOP10 cells with a One Shot Chemical Transformation kit (Invitrogen). For each patient sample, plasmid DNA was isolated and the insert was sequenced with the same oligonucleotides.

Immunohistochemistry. When available, formalin-fixed or snap-frozen cardiac tissues from affected patients were studied for the distribution of intercellular junction proteins with confocal immunofluorescence microscopy, as previously described (28,29). Antigens were exposed in paraffin-embedded sections with microwave antigen-recovery techniques (28,29) and then stained with commercial rabbit polyclonal antibodies against connexin 43 (Cx43) (Zymed, Invitrogen), the C-terminal domain of DSP (Serotec, Raleigh, North Carolina), a conserved sequence in the N-cadherins (Sigma-Aldrich, St. Louis, Missouri), and DES (ScyTek Laboratories, Logan, Utah) as well as mouse monoclonal antibodies against JUP (Sigma-Aldrich), DSC 2/3 (Zymed, Invitrogen), PKP-2 (Biodesign International, Saco, Maine), α-catenin (Zymed, Invitrogen), β-catenin (Zymed, Invitrogen), and the C-terminus of JUP (Research Diagnostics, Inc., Concord, Maine). The sections were then stained with appropriate secondary antibodies conjugated with CY3 and visualized by confocal microscopy as described previously (28,29). Patient samples were stained simultaneously with myocardial sections prepared from 3 age-matched control subjects with no clinical or autopsy evidence of heart disease. The amount of immunoreactive signal for each protein at the intercalated disks was evaluated in a blinded fashion and scored as being strongly present, weakly present, or absent.

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Results

Genetic analysis: *PKP2* mutations and compound heterozygosity. After informed consent, 198 probands (143 North American, 55 Italian) meeting clinical criteria for the diagnosis of ARVC with the Task Force criteria (Table 1) and/or ARVD registry criteria were enrolled in the genetic analysis study. All subjects were screened for mutations in all desmosome-encoding genes, with complete sequencing of the entire gene performed in all cases. Twenty-one variants in *PKP2* were identified in 38 of the 198 probands (19%), a frequency similar to previous reports (8,18–21). The variants identified included 8 missense, 4 nonsense, 2 splice site mutations, and 8 deletion/insertion mutations; the deletion/insertion variants each predict a protein frame shift (Table 2). Several variants were identified in multiple probands, including nonsense and frame-shift variants (Table 2). All of the missense substitutions resulted in changes at residues that are conserved between species (Supplementary Fig. 1). Among the variants identified, the splice site substitution detected in intron 10 and the 2509delA in exon 13 have been previously reported (8). The remaining variants are novel. Despite the fact that several of these variants were identified in multiple individuals, analysis of 700 ethnically matched control individuals (1,400 chromosomes) identified only 1 of these variants (A372P) in the control population (1 in 700).

Analysis of the family pedigrees shows that there is significantly reduced penetrance of these *PKP2* variants, similar to that reported by Gerull et al. (8) (Figs. 1A and 1B). However, in 9 of the probands with *PKP2* variants, we identified 2 distinct *PKP2* variants (Table 3, Patients #5, #7, #8, #11, #12, #14, #15, #16, and #17), consistent with compound heterozygosity. One of these probands (Patient #5 in Table 3, Fig. 1C) was a member of a family with a history of ARVC in his generation (Fig. 1C). The DNA was available from the living affected subject (Patient #6 in Table 3, Fig. 1C) and unaffected siblings of the proband as well as the phenotypically normal parents, paternal uncle, and paternal grandparents. All family members were clinically evaluated with the Task Force criteria (Table 1). Only the clinically affected brother carried both variants (Fig. 1C). The parents were carriers of individual *PKP2* variants, and the clinically unaffected (not meeting Task Force criteria) sibling hosted only the variant transmitted by his father and paternal grandfather (Fig. 1C). Reverse transcription PCR of *PKP2* messenger ribonucleic acid was performed with samples obtained from 5 other affected probands, and sequencing revealed that in each case the variants were encoded in *trans*. Thus, these variants were either inherited independently from the phenotypically normal patients or were de novo. As noted in Table 3, disparities between the clinical phenotypes and genetic findings seem somewhat common. Notably, all subjects had RV involvement, several had biventricular disease, and all had associated arrhythmias. Importantly, not all subjects met full Task Force criteria, thereby pointing to imperfections in these criteria and necessitating modifications of these criteria (Marcus et al. [30]).

These findings led us to consider the possibility that ARVC might be due to another form of "compound heterozygosity" with mutations/genetic variants in 2 desmo-

Table 2 ***PKP2* Gene Variants Identified in Patients With ARVC**

Nucleotide Change	Exon	Amino Acid Change	U.S. Probands	Italian Probands
145_148delCAGA	1	S50fsX110	4	3
419C>T	3	S140F	1	0
627C>G	3	S209R	0	1
630C>T	3	Q211X	1	1
1114G>C	4	A372P	3	0
1162C>T	4	R388W	1	0
1170-2A>G		Intron 4	1	0
1212insT	5	L404fsX409	1	0
1368delA	5	K456fsX458	1	0
1592T>G	7	I531S	3	0
1613G>A	7	W538X	2	0
1643delG	7	G548fsX562	0	1
1760G>A	8	V587I	0	1
1978C>T	10	Q660X	1	0
2009delC	10	N670fsX683	0	1
2119C>T	10	Q707X	0	1
2146-1G>C		Intron 10	3	0
2197_2202insGdelCACACC	11	H733fsX740	1	0
2359C>T	12	L787F	1	0
2447_2448delCC	12	T816fsX825	0	2
2509delA	13	V837fsX930	3	0

ARVC = arrhythmogenic right ventricular cardiomyopathy; *PKP2* = plakophilin-2.

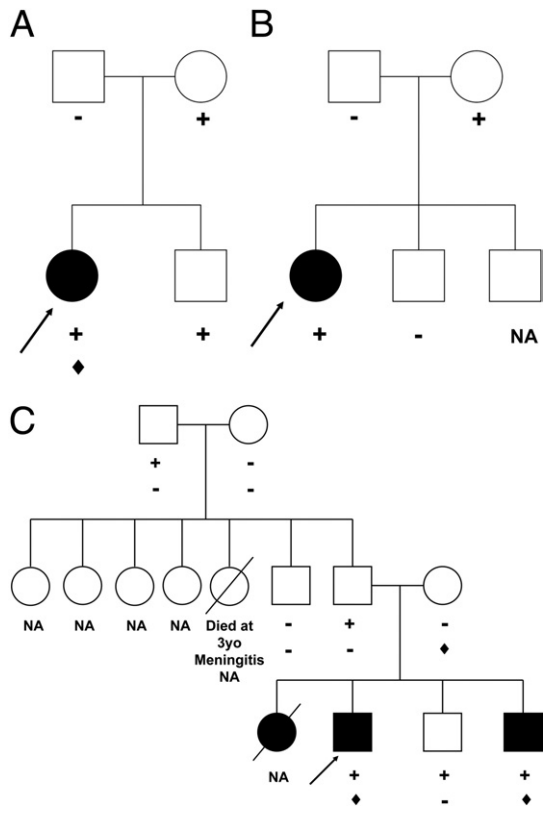

Figure 1 **Pedigrees of 3 Families With *PKP2* Variants and Incomplete Penetrance**

(A) L404fsX409 plakophilin-2 (*PKP2*) was identified in the proband and unaffected sister and was inherited from the unaffected mother **(+)**. In addition, a de novo variant, *desmoplakin* (*DSP*) Q90R, was identified in the proband **(diamond)**. The **arrow** indicates the proband. **(B)** K456NfsX458 was identified in the proband and inherited from the unaffected mother **(+)**. The **arrow** indicates the proband. **(C)** Pedigree of a family with compound heterozygous *PKP2* variants. V837fsX930 **(+)** was identified in the proband and both of his siblings and was inherited from the unaffected father, who in turn inherited it from his unaffected father. R388W **(diamonds)** was identified in the proband and his affected brother and was inherited from his phenotypically normal mother. The **arrow** indicates the proband. NA = not available for genetic analysis; yo = years old.

somal protein encoding genes required for clinical disease, called "digenic heterozygosity."

Genetic analysis: digenic heterozygosity. In all 198 probands, the desmosome-encoding genes *DSP*, *DSC2, DSG2*, *JUP*, *DSC2, DSG2, PKP4*, and *DES* were sequenced in addition to *PKP2* sequencing. This sequencing identified 13 variants in second desmosomal genes in 13 subjects with *PKP2* variants, including *DSP* in 6 cases, *DSG2* in 5 cases, *PKP4* in 1 subject, and *DSC2* in 1 subject (Table 3). None of these variants was identified in at least 700 ethnic-matched control subjects (>1,400 chromosomes). Thus, of the 38 probands with *PKP2* variants, compound or digenic heterozygosity was identified in 16 (42%), including 6 probands with 3 variants: in 2 of these cases, 1 of the *PKP2* variants was the A372P polymorphism identified in the control population. Five probands had additional family members available for clinical and genetic evaluation, including the following probands: patient 1 with the Q90R *DSP* variant (Table 3), the *DSP* W207X variant (Patient #2 in Table 3), the *DSG2* V56M variant (Patient #14 in Table 3), the DSG2 R146H variant (Patient #15 in Table 3), and the DSP R1255K variant (Patient #16 in Table 3). The *DSP* Q90R variant was a de novo substitution and therefore could be pathogenic alone or in combination with the *PKP2* variant (Fig. 1A). In Patient #12, a 4609C>T *DSP* substitution (R1537C) was also identified, a variant previously reported as a single nucleotide polymorphism. Whether this variant becomes clinically relevant in combination with the *PKP2* variants and is needed for the development of clinical features is speculation until animal models are completed. In the family of Patient #2 (Fig. 2A), the *PKP2* I531S variant was identified in 3 of the 4 siblings available for study. All family members were clinically evaluated with the Task Force criteria (Table 1), and 1 of the 3 siblings was clinically unaffected (and also has twin carrier daughters), whereas the other 2 siblings were clinically affected. Only the proband hosted the second variant, *DSP* W207X. In this case, we propose 3 potential interpretations of these data. Because the proband had the most severe phenotype (sudden death at age 25 years and severe ARVC on autopsy), it is possible that the *DSP* W207X mutation was required to manifest severe clinical disease. Second, this mutation might have no involvement in the development of disease and another as yet identified mutation is carried by the affected siblings but not by the unaffected sibling. Third, the I531S mutation might be disease-causing alone with extremely reduced penetrance, as has been described in other forms of cardiomyopathy, and requires other factors—such as acquired agents—to manifest and contribute to clinical expression. In this regard, it should be noted that the proband developed mononucleosis 2 years before death.

Patient #14 hosted a DSG2 V56M variant, as previously noted; in addition, however, this subject also had 2 additional *PKP2* variants (Fig. 2B). On the basis of evaluation of the pedigree, it is difficult to be certain as to the role of each of these variants; however, it seems that the combination of *PKP2* S140F and *DSG2* V56M, which was only detected in the proband, led to a more severe clinical phenotype (Fig. 2B).

Patient #15, a 51-year-old man, was diagnosed with a severe form of ARVC at the age of 39 years after an episode of ventricular fibrillation. He was found to host 3 different variants (*PKP2* S209R, *PKP2* T816fsX825, and *DSG2* R146H) (Fig. 3A). The *PKP2* T816fsX825 was inherited from his mother (II,2). Because his father's DNA was not available, it was not possible to establish whether the 2 missense variations (*PKP2* S209R and *DSG2* R146H) were inherited from the father or are de novo mutations. His mother and maternal aunt, who both carried the *PKP2* frame-shift variant, were clinically unaffected by Task Force criteria and were asymptomatic.

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Table 3 Clinical Demographic Data of ARVC Subjects With Desmosomal Gene Mutations

Patient #	Sex	Age Onset (yrs)	FHX	Affected Parents	Nationality	Palpitations	Syncope/ SCD	CHF/ NYHA	Arrhythmia	ECG Abnormal	SAECG Abnormal
1	F	25	N	No	U.S.	Yes	No/No	No/I	VT apex RVOT	Yes	Yes
2	F	22	Y	No	U.S.	Yes	Yes/No	No/I	VT apex RVOT	Yes	Yes
3	F	23	Y	No	U.S.	Yes	Yes/No	No/I	VT RVOT	Yes	Yes
4	M	29	N	No	U.S.	Yes	No/No	No/I	VT apex	Yes	Yes
5*	M	16	Y	No	U.S.	Yes	Yes/No	No/I	VT RVOT	Yes	Yes
6	M	12	Y	No	U.S.	Yes	Yes/No	No/I	VT RVOT	Yes	Yes
7	M	32	Y	No	U.S.	Yes	Yes/No	No/I	VT RVOT apex	Yes	Yes
8	M	52	N	No	U.S.	Yes	No/No	No/I	VT RVOT	Yes	Yes
9†	M	26	Y	No	U.S.	Yes	No/No	No/I	VT RVOT	Yes	Yes
10	F	25	Y	No	U.S.	Yes	No/No	No/I	VT RVOT	Yes	No
11	F	30	N	No	U.S.	Yes	No/No	No/I	NSVT	Yes	No
12	M	41	N	No	U.S.	Yes	Yes/No	No/I	VT	Yes	Yes
13	M	30	Y	No	U.S.	Yes	Yes/No	No/I	VT	No	Yes
14	M	50	Y	No	U.S.	Yes	Yes/Yes	No/I	VT-RV origin	Yes	Yes
15	M	44	Y	No	Italian	Yes	Yes/Yes	No/I	VF	Yes	Yes
16	M	40	Y	No	Italian	Yes	Yes/Yes	No/I	VF	Yes	No
17	F	31	Y	No	Italian	Yes	No/No	No/I	NSVT	Yes	Yes
18	M	34	Y	1	Italian	Yes	No/No	No/I	NSVT	Yes	Yes

Note that most but not all subjects with mutations meet full Task Force diagnostic criteria. All subjects have right ventricular involvement and arrhythmias, and several subjects also have left ventricular involvement. *Patients #5 (proband) and #6 are from the same family; †Patients #9 (proband) and #10 are from the same family.

ARVC = arrhythmogenic right ventricular cardiomyopathy; CHF = congestive heart failure; DSC = desmocollin; DSG = desmoglein; DSP = desmoplakin; DX Criteria = diagnostic criteria; ECG = electrocardiographic; FHx = family history of arrhythmogenic right ventricular cardiomyopathy; ICD = implantable cardioverter-defibrillator; M/m = major/minor criteria; NSVT = nonsustained ventricular tachycardia; NYHA = New York Heart Association functional class; PKP = plakophilin; RVOT = right ventricular outflow tract; SAECG = signal-averaged electrocardiogram; SCD = sudden cardiac death; VT = ventricular tachycardia.

Patient #16, a 40-year-old man, was diagnosed with a severe form of ARVC after an episode of ventricular fibrillation that occurred during a sports activity. After resuscitation, he received an implantable cardioverter-defibrillator. His family history included a male cousin who died suddenly at 30 years of age while hiking. No autopsy was performed. The proband (II,1) inherited the *DSP* missense mutation and *PKP2* stop mutation from his mother (I,2) and the missense *PKP2* mutation from the father (I,1) (Fig. 3B). At least 1 mutation was also detected in the proband's sons. In this family, all mutation carriers, with the exception of the proband, were completely asymptomatic and clinically unaffected by Task Force criteria.

Overall, there was more ventricular fibrillation and exercise-induced ventricular tachycardia in those subjects having compound or digenic heterozygosity compared with those subjects with single heterozygous PKP2 mutations. In addition, the age of onset of symptomatic ARVC, most commonly ventricular arrhythmias or syncope, was earlier in those subjects with multiple genetic variants in PKP2 or in PKP2 plus other desmosomal genes (mean age 31.5 years) compared with those subjects with single heterozygous gene mutations (mean age 39.5 years). Therefore, it seems that compound and digenic heterozygosity leads to more clinically apparent and severe ARVC with an earlier age of onset than those hosting heterozygous mutations in these genes.

Genetic analysis: single gene mutations. In 14 of the 198 subjects analyzed (7%), single heterozygous mutations were identified in desmosome-encoding genes other than *PKP2*. The heterozygous mutations identified included 4 variants in *DSP*, 5 variants in *DSG2*, 3 variants in *DSC2*, and 2 variants in *JUP* (Table 4). All variants were identified in conserved regions, and none of these variants were identified in 700 ethnic-matched control subjects (1,400 chromosomes).

Functional analysis. Myocardial autopsy samples were available from 2 individuals with *PKP2* variants, including 1 patient (PG) with a single heterozygous *PKP2* W528X nonsense mutation (no second variant has been identified to date) and a second patient (M686) with digenic heterozygosity including the *PKP2* I531S variant as well as a *DSP* W207X mutation. These myocardial samples were sectioned and stained for a variety of junctional proteins, including PKP2, DSP, JUP, N-cadherin, and DSC2/3, as well as for Cx43 and DES (Fig. 4). Staining of samples from patient PG was very weak or absent for each of these proteins except for DSP, which was normal. In contrast, staining of samples from patient M686 was essentially normal for each antibody, except for Cx43, which was absent (Fig. 4), and DSP, which seemed to stain weakly. These data suggest that the DSP nonsense mutation in Patient M686 does not affect DSP localization and that these variants do not grossly affect the desmosomal junctions. In contrast, both the desmosomal and cadherin junctions seem to be affected in patient PG, with loss of staining for N-cadherin and JUP, in addition to PKP2. Signal intensities were comparable in samples obtained from both LV and RV and in areas relatively unaffected by fibrofatty replacement.

Table 3 Continued

ICD	Transplant	Exercise VT	RV Involvement	LV Involvement	MRI Abnormal	DX Criteria M/m	Histology	Gene	Nucleotide Change	Amino Acid Change
No	No	No	Yes	No	Yes	Yes/Yes	Yes	*PKP2 DSP*	1212insT 269A>G	L404fsX409 Q90R
Yes	No	No	Yes	No	Yes	Yes/Yes	Yes	*PKP2 DSP*	1592T>G 620G>A	I531S W207X
Yes	No	Yes	Yes	Yes	Yes	Yes/Yes	Yes	*PKP2 DSG2*	1592T>G 146G>A	I531S R49H
No	No	Yes	Yes	No	Yes	Yes/Yes	No	*PKP2 DSG2*	145_148delCAGA 1003A>G	S50fsX110 T335A
Yes	No	No	Yes	No	Yes	Yes/Yes	Yes	*PKP2*	1162C>T	R388W
Yes	No	No	Yes	No	Yes	Yes/Yes	Yes	*PKP2*	2509delA	V837fsX930
Yes	Yes	Yes	Yes	No	Yes	Yes/Yes	Yes	*PKP2 PKP2*	145_148delCAGA 1592T>G	S50fsX110 I531S
Yes	No	No	Yes	Yes	Yes	Yes/Yes	No	*PKP2 PKP2*	1592T>G 2359C>T	I531S L787F
Yes	No	Yes	Yes	No	Yes	Yes/Yes	Yes	*PKP2*	1613G>A	W538X
Yes	No	No	Yes	No	Yes	No/Yes	Yes	*DSC2*	1914G>C	Q63SH
Yes	No	Yes	Yes	No	Yes	No/Yes	No	*PKP2 PKP2 DSP*	1613G>A 1914G>C 88G>A	W538X Q638H V30M
Yes	No	Yes	Yes	No	Yes	Yes/No	No	*PKP2 PKP2 DSP*	1114G>C 2145-1G>C 4609C>T	A372P R1537C
Yes	Yes	Yes	Yes	No	Yes	Yes/No	Yes	*PKP2 PKP4*	2146-1G>C 2786A>G	D929G
Yes	No	Yes	Yes	No	Yes	Yes/Yes	Yes	*PKP2 PKP2 DSG2*	419C>T 2146-1G>C 166 G>A	S140F V56M
Yes	No	No	Yes	No	Not performed	1/3	No	*PKP2 PKP2 DSG2*	627C>G 2447_2448delCC 437G>A	S209R T816fsX825 R146H
Yes	No	Yes	Yes	No	Yes	1/3	No	*PKP2 PKP2 DSP*	630C>T 2333T>C 3764G>A	Q211X I778T R1255K
No	No	No	Yes	Yes	Not performed	1/4	Yes	*PKP2 PKP2 DSP*	2119C>T 184C>A 4961T>C	Q707X Q62K L1654P
No	No	No	Yes	No	Not performed	1/4	No	*PKP2 DSG2*	145_148delCAGA 1115G>A	S50fsX110 V391I

Discussion

Arrhythmogenic right ventricular cardiomyopathy has emerged as a significant cause of sudden death, heart failure, and the need for heart transplantation over the past decade (2,31). The incidence was initially thought to be particularly high in the Veneto region of Italy and in other parts of Europe but low elsewhere (2). More recently, the disease has been increasingly recognized throughout the world (3,32,33). It is believed to be inherited in a moderate percentage of cases, with autosomal dominant inheritance predominating (4). Multiple genes, mostly those encoding desmosomal proteins, have been identified as causative in ARVC, with *PKP2* reported to be responsible for approximately 25% of all cases (8,18–21). In the work presented herein, however, interpretation of these data is questioned, and the concepts of low penetrance as well as compound and digenic heterozygosity are proposed as potential determinants of the clinical presentation in subjects carrying mutations and in their family members. In addition, identification of novel heterozygous mutations in other desmosome-encoding genes, including novel mutations in *JUP*, further supports the notion that the "final common pathway" for ARVC is the cell–cell junctions (34).

In this report, we demonstrate that, whereas variants in *PKP2* are relatively common (identified in 38 of 198 or 19% of probands), harboring 1 *PKP2* variant might not by itself be sufficient to determine overt clinical disease. In at least 16 of 38 (42%) cases, concomitant causes such as either a "second hit" in the same gene (compound heterozygosity) or in a second desmosome-encoding gene (digenic heterozygosity) or an acquired disruption of these proteins or environmental factors have been shown to be required for the overt clinical phenotype to develop or for modification of disease severity. Hence, whereas *PKP2* "mutations" are relatively common, a second variant in *PKP2* or in *DSP*, *DSC2*, *DSG2*, *PKP4*, *JUP*, or another interacting junction protein-encoding gene seems to be important for the disease and its clinical consequences to be manifest. Other "second hit" genes or interactors are likely to be discovered in the future. In addition, a variety of heterozygous mutations in all of the known desmosome-encoding genes were identified to cause ARVC and its associated clinical signs and symptoms.

Interestingly, the subjects harboring more than 1 variant in PKP2 and/or PKP2 plus other desmosome-encoding genes seem to have earlier onset of disease and more clinical severity than those individuals harboring heterozygous mutations alone. Interpretation of these data, however, is confounded by the small number of large pedigrees available for analysis. To track segregation of the genotype with disease, large families with clinical ARVC are required, but in our study, small families and sporadic cases were predominantly seen. However, in the families in which multiple individuals were enrolled and genetically screened, a single genetic variant commonly did not lead to overt clinical disease with the Task Force criteria (Table 1) or the stringent diagnostic evaluation used by the ARVD registry. It will be interesting to determine whether the carriers previously phenotyped as unaffected will be found to have

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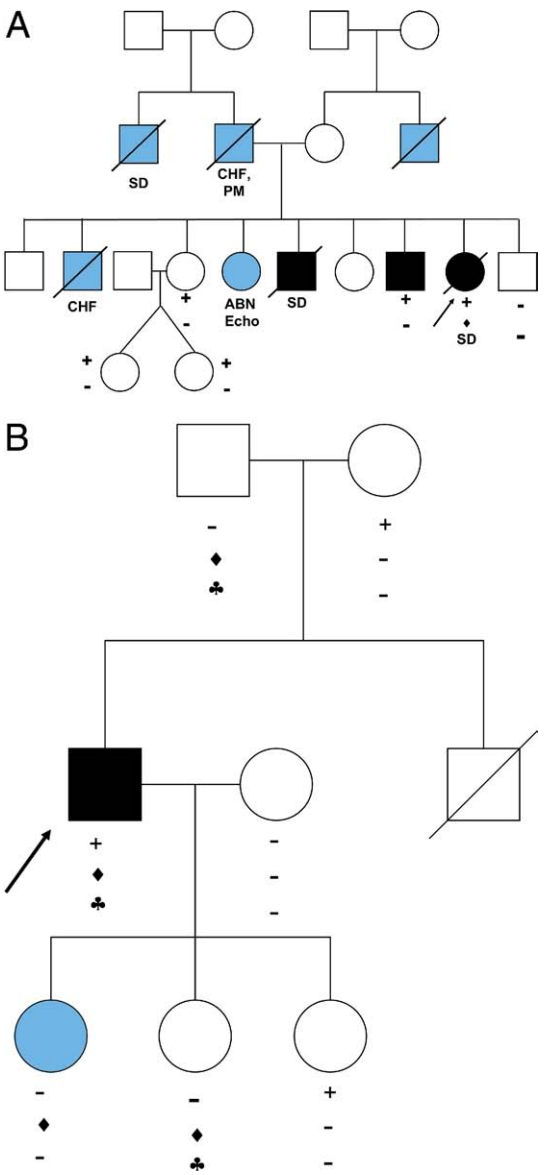

Figure 2 **Pedigree of 2 U.S. Families With Compound Desmosomal Mutations (Digenic Heterozygosity)**

(A) *PKP2* I531S (+) was identified in the proband and 2 of her siblings. *DSP* W207X **(solid diamonds)** was identified in the proband who experienced sudden death at 25 years of age. Her affected living brother was diagnosed by cardiac magnetic resonance imaging and is still alive at the age of 42 years. Her unaffected sister carrying the I531S variant is now 51 years of age and has twin unaffected daughters, age 19 years, both of whom carry the variant. Individuals without a definitive diagnosis of arrhythmogenic right ventricular cardiomyopathy (ARVC) but in whom signs or symptoms compatible with this diagnosis were reported are identified in **blue**. The **arrow** indicates the proband. **(B)** *PKP2* S140F (+), *PKP2* IVS10-1G>C **(diamonds)** and *desmoglein-2 (DSG2)* V56M **(club symbols)** were detected in the proband. The oldest child of the proband, who is 19 years old, does not meet full Task Force criteria of ARVC but has arrhythmias and symptoms. The **arrow** indicates the proband. ABN Echo = abnormal echocardiogram; CHF = congestive heart failure; PM = pacemaker; SD = sudden death; other abbreviations as in Figure 1.

any signs of early ARVC upon detailed examination (MRI, echocardiogram, and the like) or whether later development of clinical signs due to an otherwise concealed pathological process will occur over time. These genetic findings should encourage physicians caring for patients with ARVC to screen as many families as possible, irrespective of clinical presentation. Clearly, longitudinal follow-up of patients and carrier family members will be critical in determining the influence of these gene variants and, more importantly, will be critical in providing excellent preventive care. As noted, we have only identified second variants in approximately 40% of the probands in whom *PKP2* variants were identified. What about the remainder? Firstly, many genes that encode proteins that either directly or indirectly contribute to the function and integrity of cell adhesion junctions remain to be screened, such as the genes encoding α- or β-catenin. Mutations in proteins of the myocyte cytoarchitecture that are linked to these proteins—such as actin, α-actinin-2, metavinculin, or Z-disk proteins—could be

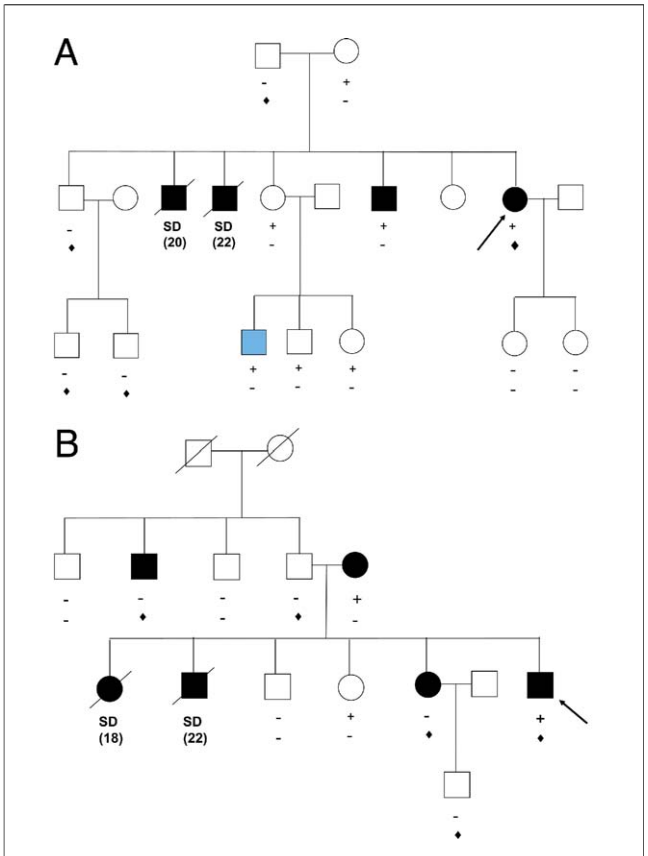

Figure 3 **Pedigree of 2 Italian Families With Compound Desmosomal Mutations**

(A) *PKP2* T816fsX825 **(diamonds)** was identified in the proband and 2 family members. *PKP2* S209R (+) and *DSG2* R146H **(club symbol)** were also identified in the proband. **(B)** *PKP2* Q211X (+), *PKP2* I778T **(club symbol)**, and *DSP* R1255K **(diamonds)** were detected in the proband. All family members carrying different mutations were completely asymptomatic. The probands are indicated by the **arrows**. SD = sudden death; other abbreviations as in Figures 1 and 2.

Table 4 Heterozygous Mutations Identified in Desmosomal Genes Other Than *PKP2*

Patient #	Sex	Gene	Nucleotide Change	Amino Acid Change
1	M	*DSC2*	874 C>T	P292S
2	M	*DSC2*	1026 A>G	I342V
3	F	*DSC2*	1660 C>T	Q554X
4	M	*DSG2*	146 G>A	R49H
5	M	*DSG2*	560 A>G	D187G
6	M	*DSG2*	1520 G>A	C507Y
7	M	*DSG2*	1003 A>G	T335A
8	F	*DSG2*	961 T>A	F321I
9	M	*DSP*	8501 G>A	R2834H
10	F	*DSP*	1598 T>C	I533T
11	M	*DSP*	688 G>A	D230N
12	F	*DSP*	1482 A>T	Y494F
13	M	*JUP*	392_394delTCA	I31dell
14	M	*JUP*	475 G>T	V159L

JUP = junctional plakoglobin; other abbreviations as in Tables 2 and 3.

important and will also be evaluated. Second, some mutations could result in dominant negative proteins giving rise to autosomal dominant inheritance, the mode of inheritance widely held as the most common in patients suffering from ARVC. In addition to the compound heterozygous and digenic mutations noted, we also identified apparent isolated heterozygous mutations in desmosome-encoding genes in another 7% of subjects. We would speculate that other relevant genes have yet to be discovered. An interesting observation from these studies is that nonsense or frame-shift mutations alone in *PKP2* often do not result in clinical disease in these families. These data would suggest that haploinsufficiency for *PKP2* is not critically important or even sufficient or that compensatory mechanisms occur. Only when the remaining copy of *PKP2* is mutated or there is a mutation in another gene does overt clinical disease develop. It also suggests that the description of these genetic variants as "disease-causing mutations" might be inaccurate and that defining affectation status by genetic analysis alone might not be entirely appropriate. For instance, on face value, the I531S variant would seem to be a polymorphism. However, the detection of this variant in 5 of the 143 probands (from different regions of the U.S.) but in none of the 700 control subjects would suggest that this variant is linked to disease. In contrast, nonsense mutations in *PKP2* would be expected to result in overt clinical disease but, in many cases, does not. For this reason, we would caution potential "fee-for-service" laboratories offering "clinical testing" for ARVC from definitive statements regarding cause-and-effect relationships, particularly in subjects in whom *PKP2* variants are identified, especially if the remaining genes are not screened. In this circumstance, not only is the affected subject at risk to have a mistaken causative gene assigned but "at-risk" family members might be either mistakenly diagnosed with a causative mutation or, more concerning, be given a negative result. In the latter case, this could lead to discharge from follow-up despite actually carrying a disease-causing mutation in another gene not analyzed. This could lead to tragic outcomes. Grossmann et al. (35) ablated the *PKP2* gene in mice, causing lethal alteration in heart morphogenesis at mid-gestation characterization by reduced trabeculations, disarrayed cytoskeleton, rupture of the cardiac wall, and hemopericardium. In the absence of PKP2, the cytoskeletal linker protein desmoplakin dissociates from the junctional plaques that connect cardiomyocytes, resulting in reduced architectural stability of intercalated disks. Constant mechanical stress, as seen in the contracting heart, is likely to be deleterious to the weakened intercellular junctions, leading to disruption, dilation, and dysfunction. However, heterozygous mice

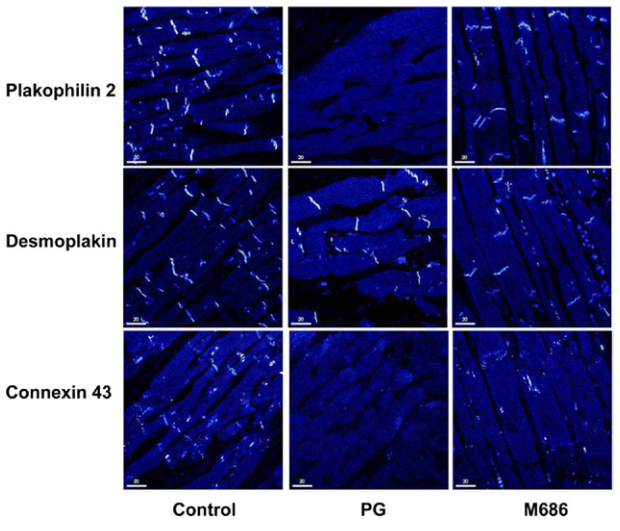

Figure 4 Immunostaining of Myocardial Sections From a Control Heart and From the Hearts of ARVC Patients PG (PKP2 W538X) and M686 (PKP2 I531S and DSP W207X)

The panels show staining for PKP2 **(top)**, DSP **(middle)**, and connexin 43 **(bottom)**. **Bars** = 20 μm. Abbreviations as in Figures 1 and 2.

were healthy and fertile. This is consistent with our notion that haploinsufficiency for *PKP2* is not sufficient to cause disease. The RV—on the basis of its geometry, architecture, and role in cardiac function—dilates to a variety of volume and pressure abnormalities and would likely develop structural and functional disease earlier than the LV. This was clearly shown in the *DSP* transgenic mouse model of Yang et al. (29), who demonstrated early RV dilation with later LV dilation as well as intercalated disk disruption and loss of desmosomes. In addition, further support is provided by the fact that the LV develops late-onset disease in some patients with ARVC (3,36). In addition to the genetic mutations, mechanical stress and stretch forces on the disturbed desmosome and intercalated disk likely play a role in disease development and severity (34,37).

Study limitations. The lack of functional studies of the variants described is a limitation of the study. Cellular and animal models incorporating these mutations individually and together could help to resolve these issues to some extent, but these approaches might not necessarily recapitulate the human condition. In an attempt to better understand the mechanisms involved in the development (or lack of development) of the clinical phenotype associated with these variants, we are in the process of developing these models for study. In addition to the models, the addition of mechanical stretch and stress on the cells and animals could facilitate the development of phenotypic differences from nonstressed models and provide greater insight.

Conclusions

Together, the genetic and functional data provided here should help to define the nature of the genetic and clinical basis of ARVC. This work could significantly impact on clinical genetic screening, because simple single gene analysis (particularly for *PKP2*) would be inappropriate, and the potential for inaccurate interpretation based on single (or even multiple) gene analysis seems to be high. More detailed information regarding the true clinical relevance of *PKP2* mutations is needed. Furthermore, the moderate number of mutations in the other desmosome-encoding genes strongly suggests that all genes in this pathway should be screened in all subjects. Identification of heterozygous mutations in *JUP* as well as the potential mutation within the *PKP4* gene adds to the spectrum of affected genes involving the desmosome. Further studies of *PKP4* in subjects with ARVC as well as functional analysis of models with mutant *PKP4* will be needed to clearly state that this is a potential disease-causing gene in ARVC.

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Key Words: arrhythmias ■ cardiomyopathies ■ desmosomes ■ intercalated disks ■ genetic mutations.

APPENDIX

For Supplementary Figure 1, please see the online version of this article.