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Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy

Pathogenic Desmosome Mutations in Index-Patients Predict Outcome of Family Screening: Dutch Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy Genotype-Phenotype Follow-Up Study

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Background—Arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C) is an autosomal dominant inherited disease with incomplete penetrance and variable expression. Causative mutations in genes encoding 5 desmosomal proteins are found in ≈50% of ARVD/C index patients. Previous genotype-phenotype relation studies involved mainly overt ARVD/C index patients, so follow-up data on relatives are scarce.

Methods and Results—One hundred forty-nine ARVD/C index patients (111 male patients; age, 49±13 years) according to 2010 Task Force criteria and 302 relatives from 93 families (282 asymptomatic; 135 male patients; age, 44±13 years) were clinically and genetically characterized. DNA analysis comprised sequencing of plakophilin-2 (*PKP2*), desmocollin-2, desmoglein-2, desmoplakin, and plakoglobin and multiplex ligation-dependent probe amplification to identify large deletions in *PKP2*. Pathogenic mutations were found in 87 index patients (58%), mainly truncating *PKP2* mutations, including 3 cases with multiple mutations. Multiplex ligation-dependent probe amplification revealed 3 *PKP2* exon deletions. ARVD/C was diagnosed in 31% of initially asymptomatic mutation-carrying relatives and 5% of initially asymptomatic relatives of index patients without mutation. Prolonged terminal activation duration was observed more than negative T waves in V_1 to V_3, especially in mutation-carrying relatives <20 years of age. In 45% of screened families, ≥1 affected relatives were identified (90% with mutations).

Conclusions—Pathogenic desmosomal gene mutations, mainly truncating *PKP2* mutations, underlie ARVD/C in the majority (58%) of Dutch index patients and even 90% of familial cases. Additional multiplex ligation-dependent probe amplification analysis contributed to discovering pathogenic mutations underlying ARVD/C. Discovering pathogenic mutations in index patients enables those relatives who have a 6-fold increased risk of ARVD/C diagnosis to be identified. Prolonged terminal activation duration seems to be a first sign of ARVD/C in young asymptomatic relatives. **(*Circulation*. 2011;123:2690-2700.)**

Key Words: arrhythmogenic right ventricular dysplasia ■ cardiomyopathy ■ desmosome ■ follow-up studies ■ genetics

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Arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C) is histopathologically characterized by progressive fibrofatty replacement of myocardium, primarily of the right ventricle (RV).[1–3] Although familial occurrence was recognized in the first report, only in the last decade has the genetic substrate been identified in genes encoding desmosomal proteins.[1,4–9] Desmosomes are protein complexes in the intercalated disk, among others responsible for mechanical coupling of cardiac myocytes. Their impairment leads to both mechanical and electric uncoupling of cardiomyocytes, followed by cell death with fibrofatty replacement.[10–13] Both uncoupling and altered architecture result in activation delay, which is the pivotal mechanism for reentry and thus ventricular tachycardia (VT).[14–16]

Editorial see p 2661
Clinical Perspective on p 2700

Arrhythmogenic right ventricular dysplasia/cardiomyopathy usually shows an autosomal dominant inheritance pattern, with incomplete penetrance and highly variable clinical expression.[1,17–20] Classically, index patients present between the second and fourth decades of life with VT originating from the RV. However, sudden death can occur as early as adolescence, whereas mutation carriers may also remain without any signs and symptoms into old age. It has been hypothesized that genetic modifiers could be responsible for this phenotypic variability.[4,5,7,21–24]

One of the primary clinical challenges in ARVD/C is timely diagnosis of the concealed phase, when individuals are at risk for arrhythmias despite the absence of symptoms. Yet, previous studies on genotype-phenotype correlations involved mainly overt ARVD/C index patients.[17–24] Follow-up data on their relatives are scarce. Hence, the proportion of relatives who develop signs of ARVD/C and/or (fatal) arrhythmias is unknown.

Analyzing multiple genes related to ARVD/C in all index patients is essential for both accurate diagnosis and appropriate family counseling and screening. More insight into the natural variability of the disease expression and phenotypic consequences of genetic findings of ARVD/C is required. We therefore sequenced all 5 desmosomal genes in 149 Dutch ARVD/C index patients. The 302 family members were screened for the pathogenic mutations identified in their respective index patients. All individuals were followed up for genotype-phenotype correlations to determine disease penetrance and expression, including arrhythmias and sudden death.

Methods

Patient Population

In total, 169 unrelated white Dutch index patients with ARVD/C diagnosed according to the recently modified diagnostic Task Force criteria (2010 TFC) or at autopsy were included.[25] An index patient was the first member of the family diagnosed with ARVD/C in whom DNA analysis was started. The diagnostic process included detailed clinical and family histories, a physical examination, a 12-lead ECG, exercise testing, chest x-ray, and 2-dimensional transthoracic echocardiography. If no VT had been recorded, 24-hour Holter monitoring was performed. Additional magnetic resonance imaging and/or left ventricular and RV cine-angiography was performed in 118 patients (70%), and electrophysiological studies were done in 105 patients (62%). See Table I in the online-only Data Supplement for details on follow-up frequencies.

We also included 302 relatives of 93 index patients who underwent cardiologic evaluation comprising at least a detailed history, physical examination, 12-lead ECG, and 2-dimensional echocardiography. If performed, outcomes of exercise tests (in 59%), 24-hour Holter monitoring (in 66%), signal-averaged ECG (in 25%), and electrophysiological studies (12%), as well as additional imaging by magnetic resonance imaging and/or RV cine angiography (42%), were included.

All 169 families received genetic counseling and consented to both clinical evaluation according to 2010 TFC and genetic screening of ARVD/C-related genes.[25] Quantitative analyses were performed at the 7 participating centers. Scoring was performed in the core laboratory in Utrecht, the Netherlands, and patients were included only if there were no signs of any other cardiac disease.

Diagnostic Criteria

Routine 12-lead ECGs were done with a paper speed of 25 mm/s and low-pass filter at 100 Hz. Depolarization and repolarization abnormalities were analyzed only while patients were off drugs. Epsilon wave was defined as a distinct deflection after the QRS complex had first returned to the isoelectric line.[26] Terminal activation duration (TAD) was determined as the longest value in V_1 to V_3, from the nadir of the S wave to the end of all depolarization deflections, and considered prolonged when $\geq$55 milliseconds.[27] Recordings of VT episodes, both spontaneous and induced by programmed electric stimulation during electrophysiological studies, were collected. Ventricular tachycardia morphologies were determined only if 12-lead ECGs were available. Tissue characterizations from biopsies were not taken into account, because analyses had not been performed as prescribed in the 2010 TFC.[25]

DNA Analysis

Genomic DNA was extracted from whole blood or paraffin-embedded tissues as described previously.[23] Sufficient DNA was available in 149 patients (88%) for direct sequencing of *PKP2*, *DSG2*, *DSC2*, *DSP*, and *JUP*. In addition, multiplex ligation-dependent probe amplification analysis was performed to identify large deletions in *PKP2* (SALSA multiplex ligation-dependent probe amplification kit P168 ARVC-PKP2, MRC Holland, Amsterdam, the Netherlands). Primer sequences and polymerase chain reaction conditions are available on request.

Nonsense, frameshift, and splice-site mutations affecting positions −2, −1, +1, and +2, as well as *PKP2* exon deletions, were all labeled truncating and considered to be proven pathogenic unless identified as polymorphisms. To assess the possible pathogenic nature of missense mutations, we used the in silico predictive programs Sorting Intolerant From Tolerant (SIFT) and Polymorphism Phenotyping (PolyPhen), which use the following criteria: difference in physicochemical properties of amino acids in respective substitutions, evolutionary conservation of amino acids across several species, presence in an evolutionary conserved region, and localization in a predicted or proven functionally important domain.[28,29] Missense mutations were considered to be most likely pathogenic when both programs predicted the genetic variants to affect protein function by a tolerance index score of $\leq$0.05 (SIFT) and the classification "probably damaging" (PolyPhen).[28,29] Variants suspected of pathogenicity also had to be absent in 200 ethnically matched (ie, white Dutch) control subjects after direct sequencing. When available, data on segregation were taken into account. Family members were screened only for the pathogenic mutation found in their respective index patient. For genotype-phenotype analyses, proven pathogenic and most likely pathogenic variants were together labeled pathogenic, and comparisons of truncating and missense mutations were made. All variants present in the general population with a frequency of >1% were considered to be sequence polymorphisms. Sequence variants that did not fulfill our criteria for pathogenicity and were not polymorphisms were labeled unclassified variants (UVs).

Table 1. Clinical Characteristics of Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy Index Patients Related to the Presence or Absence of Pathogenic Mutations

	Total (n=147)*		With Mutation (n=87)		No Mutation (n=60)		
	n	%	n	%	n	%	*P*
Male	110	75	62	71	48	80	0.284
Age at onset, mean±SD, y	37±14		35±13		40±14		0.042
Follow-up, mean±SD, y	12±9		13±10		11±7		0.191
Reason for first evaluation							
VT	122	82	72	83	47	78	
Aborted sudden death	12	8	8	9	4	7	
Prolonged syncope	2	1	1	1	1	2	
Sudden death of a relative	2	1	0	0	2	3	
Frequent PVCs	5	3	5	6	3	5	
Other	4	3	1	1	3	5	
TFC							
Epsilon wave†	22	18	14	18	8	17	0.153
Late potentials‡	44	54	22	50	22	59	0.395
Prolonged TAD†	78	62	44	56	34	72	0.734
Negative T waves							
V_1–V_3†	98	78	69	88	29	62	0.001
V_1–V_2†	8	6	5	6	3	6	1.000
V_4–V_6†	6	5	2	3	3	6	0.524
Ventricular fibrillation	12	8	8	9	4	7	0.281
LBBB VT with superior axis	67	46	39	45	28	47	0.943
LBBB VT	129	88	77	89	52	87	0.945
PVCs >500/24 h	33	22	20	23	13	22	0.969
Structural major TFC	86	59	55	63	31	52	0.198
Structural minor TFC	25	17	15	17	10	17	0.963

VT indicates ventricular tachycardia; PVC, premature ventricular complex; TFC, Task Force criteria; TAD, terminal activation duration; and LBBB, left bundle-branch block. *P* values are the difference between mutation carriers and noncarriers.

*Two patients were diagnosed after autopsy; therefore, no clinical data were available.

†ECGs done while the patients were off drugs were available for 125 index patients: 78 with and 47 without mutation.

‡Late potentials were measured in 81 patients: 69 by signal-averaged ECG and 12 by mapping during electrophysiological studies.

In this article, "patients with mutations" and "mutation carriers" refer to patients carrying pathogenic mutations as identified in this study.

Statistical Analysis

Continuous variables were compared by use of the Student *t* test. Categorical variables were analyzed by use of contingency tables and the Pearson χ^2 method. If the expected value was <5, the Fisher exact test was used instead. To compensate for possible correlation of characteristics of relatives within families, mixed models with hierarchical structure of members within families were applied. When it was impossible to fit the data in these models owing to lack of variation within families, Pearson χ^2 tests were applied instead on a family level (ie, on the presence/absence of characteristics within separate families). Descriptive statistics are reported as mean±SD and estimates as estimated value±SE. Values of $P<0.05$ were considered statistically significant. PASW statistics 17.0 software (SPSS, Chicago, IL) was used for calculations.

Results

Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy Index Patients

Initially, 169 ARVD/C patients fulfilled the 2010 TFC. Twenty patients were excluded because of incomplete DNA analyses. *PKP2*, *DSG2*, *DSC2*, *DSP*, and *JUP* were all screened in the remaining 149 patients (111 male patients; mean age at inclusion, 49±13 years). First presentation was at a median age of 37 years (range, 12 to 77 years), mostly with monomorphic VT (n=122, 82%). These VTs were sustained in 119 cases, and all but 2 had left bundle-branch block morphology (see Table 1). Presenting first symptoms were similar between men and women. Cardioverter-defibrillators were implanted in 95 patients (64%): in 57 directly after diagnosis and in 38 after 4±7 years of follow-up. During follow-up, 4 patients died of ventricular fibrillation (3 without implantable cardioverter-defibrillators, 1 with electrical storm) and 2 died of progressive heart failure. Three patients were indicated for heart transplantation; 1 received a transplantation.

DNA Analyses

All pathogenic mutations are summarized in Table 2, and UVs are summarized in Table II in the online-only Data Supplement. In total, 87 index patients (58%) carried at least 1 mutation (89 total: 70 truncating [68 *PKP2*] and 19 missense [12 *PKP2*]).

Table 2. Pathogenic Mutations

Gene	DNA Change	Protein Change	Type	PolyPhen (PSIC)*	SIFT†	Index Patients, n	Family Members Symptomatic, n	
							Yes	No
Proven pathogenic								
PKP2	Deletion exon 1–4	NA	Deletion			1	1	7
	Deletion exon 1–14	NA	Deletion			1		4
	Deletion exon 8	NA	Deletion			1		
	c.148_151delACAG	p.Thr50SerfsX61	Frameshift			2		3
	c.235C>T	p.Arg79X	Nonsense			9	3	15
	c.258T>G	p.Tyr86X	Nonsense			1		
	c.397C>T	p.Gln133X	Nonsense			9	3	16
	c.917_918delCC	p.Pro318GlnfsX29	Frameshift			3	1	1
	c.1211_1212insT	p.Val406SerfsX	Frameshift			11	6	15
	c.1369_1372delCAAA	p.Gln457X	Nonsense			2		4
	c.1848C>A	p.Tyr616X	Nonsense			4		6
	c.2028G>A	p.Trp676X	Nonsense			1		1
	c.2034G>A	p.Trp678X	Nonsense			1		2
	c.2146-1G>C		Splice site			7		2
	c.2386T>C	p.Cys796Arg	Missense	++ (3.410)	0.03	11	1	17
	c.2421C>A	p.Tyr807X	Nonsense			1		
	c.2489+1G>A		Splice site			6	1	7
	c.2489+4A>C†		Splice site			4		9
	c.2509delA	p.Ser837ValfsX94	Frameshift			1		
	c.2544G>A	p.Trp848X	Nonsense			1	1	
DSC2	c.943-1G>A		Splice site			1		1
DSG2	c.378+2T>G		Splice site			1		
DSP	c.3337C>T	p.Arg1113X	Nonsense			1	1	
Most likely pathogenic								
PKP2	c.2062T>C	p.Ser688Pro	Missense	+	0.04	1		1
DSC2	c.608G>A	p.Arg203His	Missense	++ (2.295)	0.00‡	1		2
	c.942+3A>G§		Splice site			1		
	c.2587G>A	p.Gly863Arg	Missense	++ (2.492)	0.00‡	1		
DSG2	c.137G>A	p.Arg46Gln	Missense	++ (2.013)	0.00‡	2		4
	c.614C>T	p.Pro205Leu	Missense	++ (3.054)	0.00‡	1		
	c.874C>T	p.Arg292Cys	Missense	++ (2.759)	0.00‡	1		2
DSP	c.1982A>T	p.Asn661Ile	Missense	++ (2.073)	0.01‡	1		

*Polymorphism Phenotyping (PolyPhen) prediction: ++, probably damaging; +, possibly damaging; −, benign. PSIC indicates Position-Specific Independent Counts.

†Sorting Intolerant From Tolerant (SIFT) prediction: Amino acids with scores<0.05 are predicted to be deleterious. For previous reports on pathogenicity, see Reference 31.

‡This substitution may have been predicted to affect function just because the sequences used were not diverse enough. There is low confidence in this prediction.

§The 4 splice-prediction programs used (SpliceSiteFinder, MaxEntScan, NNSPLICE, and GeneSplicer) indicated disruption of the splice donor site of *DSC2* exon 7.

Single mutations were identified in 84 index patients (56%): 76 *PKP2* (64 truncating), 5 *DSG2*, 2 *DSC2*, and 1 *DSP*. In addition, 3 patients (2%) carried multiple mutations. Two had a truncating *PKP2* mutation and a *DSC2* or *DSP* missense mutation, and 1 carried a homozygous *DSC2* mutation (see Table III in the online-only Data Supplement for details, including phenotypic characteristics).

We identified 52 UVs in 50 patients (36 different ones; Table II in the online-only Data Supplement). In 28 patients, the UV was carried in addition to a pathogenic mutation, and 2 patients carried 2 UVs. Table IV in the online-only Data Supplement shows the combinations of pathogenic mutations and UVs per patient. Of the 36 UVs, 29 were missense variants, 6 were silent variants, and 1 was a frameshift variant. The *DSC2* frameshift variant p.Ala897fs was considered a UV because it is located at the far end of the gene and was found in 8 patients and in 3 of 200 controls ($P=0.06$).[24] The *DSG2* variant p.Val158Gly was predicted to be patho-

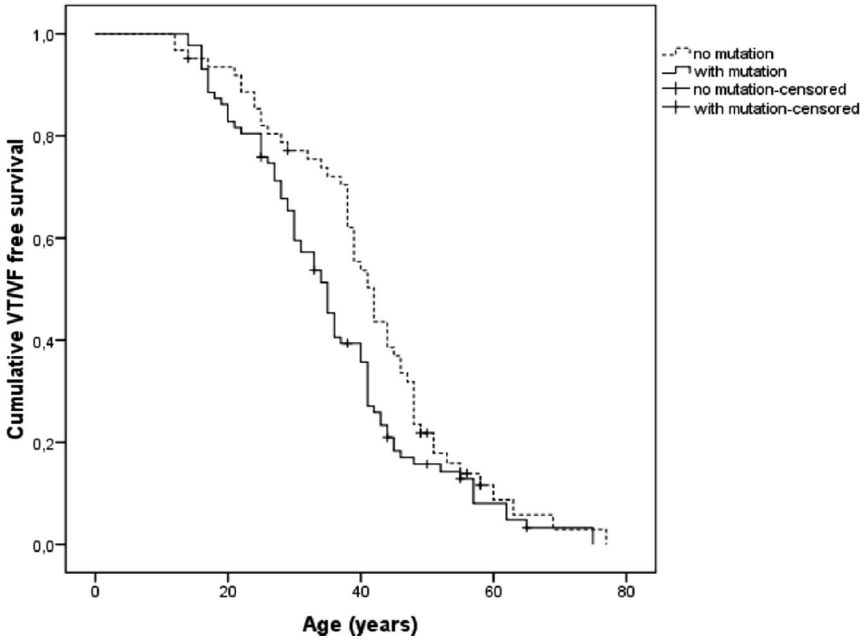

Figure 1. Ventricular tachycardia/ventricular fibrillation (VT/VF)–free survival of arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C) index patients with vs without mutations. Patients with a mutation experience their first arrhythmic event at a significantly younger age than those without a mutation. This age is even lower if a patient carries 2 mutations (see text).

genic by both SIFT and PolyPhen. However, we found this variant in 2 of 149 patients and 3 of 300 controls ($P=0.67$), and it has frequently been reported in other control populations.[22,23,30,31] Therefore, we classified it as a UV.

Genotype-Phenotype Correlation

First, we compared the clinical characteristics of index patients with and without mutations independently of the number of variants (Table 1). Both groups showed a male predominance (71% and 80%), and age at the time of inclusion was similar (51±13 and 49±13 years).

Ventricular tachycardia and/or ventricular fibrillation was recorded in 134 of 149 index patients, showing similar frequencies among those with and without mutations. However, first arrhythmic events were documented at a significantly younger age in mutation carriers compared with noncarriers (median age, 35 versus 42 years; $P=0.042$; Figure 1). The 3 patients carrying 2 mutations had their first VT at 14, 17, and 20 years of age. A comparison of criteria in mutation carriers and noncarriers demonstrated no significant differences except that negative T waves in V_1 to V_3 occurred more often in mutation carriers (88% versus 62%; $P=0.001$). None of the criteria could be explained by the type of mutation (truncating versus missense) except for prolonged TAD, which was observed more often in patients with missense mutations (13 of 15 versus 31 of 63).

Patients carrying a single UV did not differ from patients without mutations on any criterion or on age of first event. However, fewer patients with a single UV had negative T waves in V_1 to V_3 than patients with 1 mutation ($P=0.043$), but they were similar in every other respect. Likewise, patients carrying a UV together with a mutation did not differ from either patients with a single mutation or those with 2 mutations (see Table V in the online-only Data Supplement for more details).

Family History of Sudden Cardiac Death

Twenty-five index patients reported 30 relatives with sudden cardiac death (SCD). In 18 families of index patients with mutations, SCD had occurred in 19 relatives (16 male subjects). Autopsy was performed in 6 cases and revealed ARVD/C in all of them. In 7 families of index patients without mutations, SCD had occurred in 11 relatives (8 male relatives). No autopsies had been performed. Mean ages at SCD were similar in families with and without mutations (28 years [range, 15 to 49 years] versus 32 years [range, 21 to 43 years]; $P=0.108$). However, all 7 relatives with SCD at <20 years of age (23%) belonged to families with *PKP2* mutations (6 truncating; Table 3).

Family Members

Both genotypic and phenotypic data were available for analysis of 302 members from 93 different families (58 with and 35 without mutations).

Relatives Symptomatic at First Presentation

Twenty relatives from 18 families presented with cardiac symptoms before family screening was performed (13 men; mean age, 42 years [range, 20 to 71 years]). In 10 relatives, monomorphic left bundle-branch block VT was the first symptom. In addition, 4 family members had ventricular fibrillation (1 was successfully resuscitated), 3 had prolonged syncope, 2 had palpitations and 1 had atrial fibrillation. Eighteen relatives fulfilled the 2010 TFC, and 18 carried pathogenic mutations (all *PKP2,* 16 truncating; Table 2 and Figure 2).

Family Screening of Asymptomatic Relatives

The remaining 282 asymptomatic relatives (123 male subjects, 44%) were evaluated in the course of family screening. Mean age at first clinical examination was 39±18 years, and follow-up 4±4 years, which was similar for male and female subjects. In 119 members (42%) from 56 families, pathogenic

Table 3. Relatives <20 Years of Age With Sudden Death or Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy Signs

	Age, y	Sex	*PKP2* Mutation in Family†	*PKP2* Mutation†	Autopsy	TFC Identified
Sudden cardiac death	15	Male	c.235C>T		No	
	16	Male	c.2489+1G>A‡		No	
	16	Male	c.2489+1G>A‡		No	
	17	Male	c.2386T>C		Yes (ARVD/C)	
	17	Male	c.397C>T		No	
	17	Male	c.1211-1212insT		Yes (ARVD/C)	
	18	Female	c.2421C>A		Yes (ARVD/C)	
Family screening*	14	Male		Deletion exons 1–4		Prolonged TAD
	17	Male		c.1211-1212insT		Prolonged TAD plus >500 PVCs/24 h
	18	Male		c.2489+4A>C		Prolonged TAD
	18	Male		c.2386T>C		Prolonged TAD
	19	Female		c.148_151delACAG		Prolonged TAD
	15	Male		c.1369_1372delCAAA		Negative T waves in V_1–V_2
	19	Female		c.2386T>C		Negative T waves in V_1–V_2

TFC indicates Task Force criteria; ARVD/C, arrhythmogenic right ventricular dysplasia/cardiomyopathy; TAD, terminal activation duration; and PVC, premature ventricular complex.

*Age after follow-up.

†All mutations in *PKP2*.

‡From the same family.

Mutations were found in none of the other genes. All mutations were truncating, except missense c.2386T>C.

mutations were identified: in 111 *PKP2* (93%; 78% truncating), 5 *DSG2*, and 3 *DSC2* (Table 2 and Figure 2).

Table 4 shows the results of family screening in the 282 initially asymptomatic family members. Most criteria were perceived either exclusively or considerably more often in mutation carriers. Because of the low presence of characteristics and low variation in families, mixed models could not be applied to these data. To recognize the possible dependence of relatives and their phenotypes among families, statistical analyses were performed at the family level (ie, families were scored positive

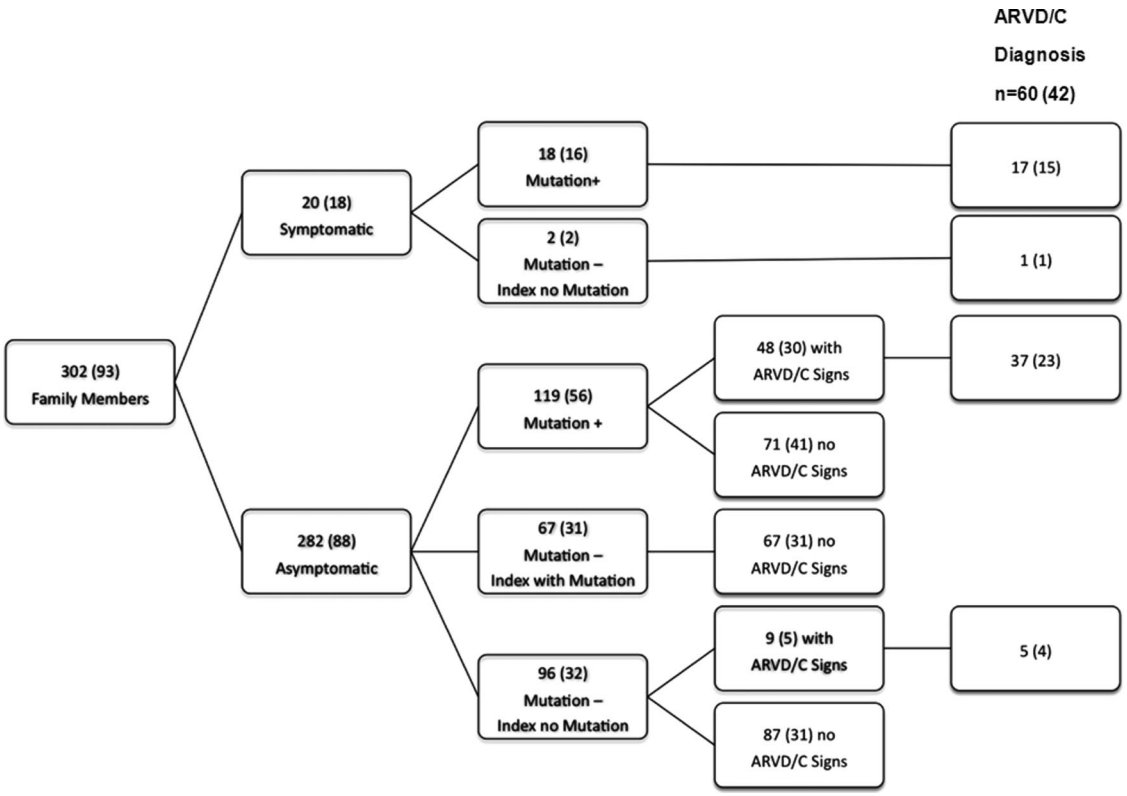

Figure 2. Schematic representation of relatives demonstrating the distribution of arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C) signs and symptoms as well as mutations. Numbers in parentheses indicate the number of different families.

Table 4. Clinical Characteristics of Asymptomatic Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy Relatives Related to the Presence or Absence of Pathogenic Mutations

	Total (n=282)		With Mutation (n=119)		No Mutation (Index Patient With Mutation; n=67)		No Mutation (Index Patient No Mutation; n=96)		
	n	%	n	%	n	%	n	%	*P**
Male	123	44	45	38	32	48	47	49	0.096
Age at first evaluation, mean±, y	39±18		39±18		40±18		38±17		0.419
Follow-up, mean±SD, y	4±4		4±3		4±3		3±3		0.131
ARVD/C diagnosis	42 (27)	15	37 (23)	31	0	0	5 (4)	5	0.004
Epsilon wave	4 (4)	1	4 (4)	3	0	0	0	0	0.292
Late potentials†	8 (8)	10	6 (6)	21	0	0	2 (2)	2	0.047
Prolonged TAD	34 (26)	12	26 (21)	22	0	0	8 (5)	8	0.030
Negative T waves									
V_1–V_3	20 (18)	7	19 (17)	16	0	0	1	1	0.002
V_1–V_2	7 (6)	2	6 (5)	5	0	0	1	1	0.410
V_4–V_6	3 (2)	1	0	0	0	0	3 (2)	3	0.130
VT with LBBB morphology									
With superior axis	2 (2)	1	2 (2)	2	0	0	0	0	0.532
With inferior axis	9 (9)	3	9 (9)	8	0	0	0	0	0.023
>500 PVCs/24 h	34 (19)	12	23 (14)	19	0	0	9 (5)	9	0.304
Structural abnormalities									
Major	12 (11)	4	10 (9)	8	0	0	2 (2)	2	0.315
Minor	10 (7)	4	10 (7)	8	0	0	0	0	0.045

ARVD/C indicates arrhythmogenic right ventricular dysplasia/cardiomyopathy; TAD, terminal activation duration; VT, ventricular tachycardia; LBBB, left bundle-branch block; PVC, premature ventricular complex.

*Difference between families of relatives with mutation vs relatives of index patients without mutation. Numbers in parentheses indicate the numbers of different families.

†Late potentials were measured in 77 patients: 29 with and 48 without mutation.

for separate criteria if present in ≥1 relative; Table 4). Strikingly, prolonged TAD was observed in more cases than negative T waves in V_1 to V_3. This difference varied with age (Figure 3). Relatives <20 years of age had no negative T waves in V_1 to V_3, but prolonged TAD was already observed in 5 of 7 young relatives with signs of ARVD/C (Table 3).

VTs were recorded in 9 initially asymptomatic relatives (3%) from 8 families. All carried *PKP2* mutations (7 truncating; Table 4). Five women (mean age, 51 years [range, 34 to 70 years]) had hemodynamically well-tolerated nonsustained left bundle-branch block VT during their first exercise tests. The other 4 (1 female) had VT after a mean follow-up of 4 years (2 to 8 years at a mean age of 43 years [range, 34 to 56 years]. Two left bundle-branch block VTs were sustained with rates of 220 and 240 bpm, and resulted in syncope.

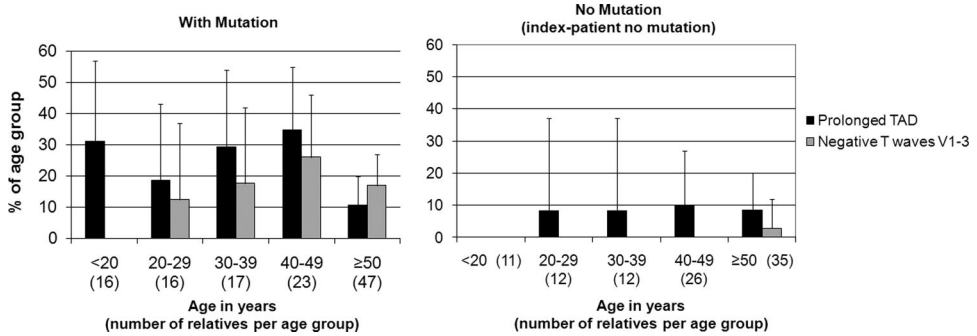

Figure 3. Presence of prolonged terminal activation duration (TAD) and negative T waves in V_1 to V_3 per age group in relatives with mutation vs relatives from index patients without mutation. Prolonged TAD was already present in many young relatives, with similar frequencies among all age groups (P=0.269). On the contrary, negative T waves were present more frequently with increasing age, almost exclusively in mutation carriers. Numbers in parentheses indicate the numbers of relatives per age group.

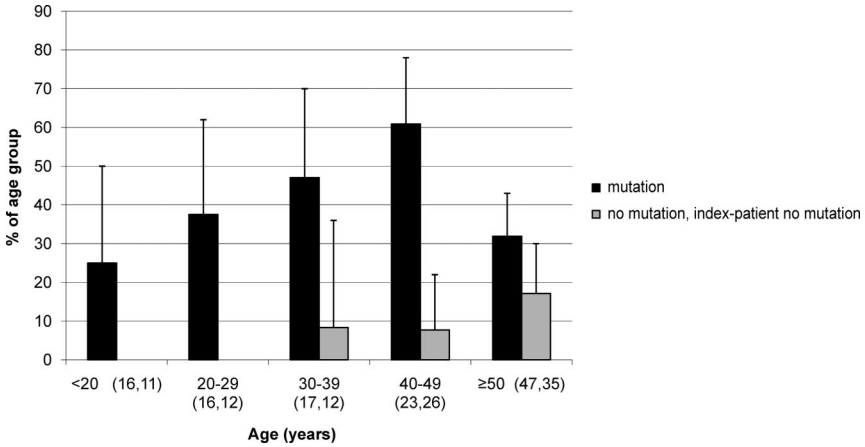

Figure 4. Relatives showing any sign of arrhythmogenic right ventricular dysplasia/cardiomyopathy, ie, meeting ≥1 criteria besides those concerning family history, divided into age groups. With increasing age, higher percentages of relatives showed signs of disease except in at the highest age. Numbers between parentheses indicate numbers of relatives per age group, with and without mutation, respectively.

Altogether, 57 family members (20%) from 35 families showed signs of ARVD/C because they fulfilled ≥1 criteria besides those concerning family history. This group comprised more women than men (39 versus 18; $P=0.04$), and their mean age after follow-up was 47±16 years. Forty-eight (84%) carried pathogenic mutations (all *PKP2*, 41 truncating), and the remaining 9 belonged to families with no identified mutation. Fourteen relatives with an unremarkable first clinical evaluation developed signs of ARVD/C after a mean follow-up of 4.2 years (range, 1 to 10 years). For family members up to 50 years of age, a higher age was associated with an increased prevalence of ARVD/C signs, in contrast to family members ≥50 years of age (Figure 4).

Forty-two initially asymptomatic family members from 27 families were diagnosed with ARVD/C: at first evaluation in 34 patients and after a mean follow-up of 5.0 years (range, 2 to 10 years) in 8 patients. This group had a mean age at diagnosis of 39±15 years and comprised 29 females (69%).

Familial Risk of Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy

In initially asymptomatic relatives, ARVD/C was diagnosed in 5% (5 of 96) when their index patient had no identified mutation and 20% (37 of 119+67) when their index patient had a mutation; all 37 also carried mutations (Figure 2). Thus, identifying a mutation in an index patient implies a relative risk for ARVD/C diagnosis in their asymptomatic relatives of 3.8±1.6. This risk increases to as much as 6.0±2.4 for mutation-carrying relatives compared with relatives of index patients without a mutation (37 of 119 versus 5 of 96).

When the symptomatic and asymptomatic mutation-carrying relatives were combined, 54 of 137 (39%) had an ARVD/C diagnosis compared with 6 of 98 relatives of index patients without mutations (6%).

Of the 93 families in which ≥2 members, including the index patient, were examined, 42 had familial ARVD/C (45%; 38 with mutations, all *PKP2*, 33 truncating). Thus, *PKP2* mutations were identified in 90% of familial ARVD/C cases.

Discussion

Here, we report the results of follow-up of a large number of ARVD/C families, including comprehensive DNA analysis. In 87 of 149 ARVD/C index patients (58%), pathogenic mutations were identified, predominantly truncating *PKP2*. ARVD/C was diagnosed in 60 of 302 family members (18 symptomatic, 42 asymptomatic), of whom 54 (90%) carried pathogenic mutations (all *PKP2*, 47 truncating). Identifying a pathogenic mutation in an index patient predicts outcome in relatives. Compared with relatives of index patients without mutations, mutation-carrying relatives have a 6-fold risk of ARVD/C diagnosis, markedly enhanced risk of ventricular arrhythmias in analyzed relatives, and earlier onset of ARVD/C signs and symptoms (for concurrent numbers, see Familial Risk of Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy, Table 4, and Figure 4, respectively). In young relatives <20 years of age, sudden death and signs of ARVD/C occurred exclusively in *PKP2* mutation carriers. Prolonged TAD, a marker of activation delay, appeared to be an early ARVD/C sign.

Pathogenicity of DNA Variants

The large majority of DNA variations were truncating *PKP2* mutations. Notably, in this first study applying multiplex ligation-dependent probe amplification on a large scale in ARVD/C patients, large *PKP2* deletions were identified in 3 cases. This mutational yield of 2%, which is comparable to the sequencing of *DSG2* or *DSC2*, underscores the importance of performing this additional analysis. Notably, because this has not been tested, we cannot exclude the possibility that large deletions might be present in other desmosomal genes in patients that are as yet without a pathogenic mutation.

The fact that a significant proportion of genetic variants remains unclassified represents a gap in risk assessment for index patients, and UVs are noninformative for family screening. However, establishing pathogenic effects of missense variants is difficult and requires well-validated functional assays, which are not widely available and are highly complex to perform. Linkage and segregational studies can be helpful to establish pathogenicity. Yet, low frequency of variants, small family size, and age-dependent penetrance hamper the use of these methods.

Previous studies on mutations in *DSC2*, *DSG2*, and *DSP* used different criteria to define the pathogenicity of DNA variants. The largest studies used criteria of absence in control subjects and/or occurrence in a functionally important domain, alteration of conserved amino acids, or cosegrega-

tion with disease in a family.[21,22,30] Thus, in contrast to our study, none of these studies used all the possible predictive strategies together. Consequently, the variants p.Thr335Ala and p.Val392Ile in *DSG2* and p.Gln90Arg in *DSP* were previously classified as pathogenic, but with our definition, they were classified as UVs.[21,22]

Index Patients

In this study of white Dutch patients, *PKP2* was by far the most important contributor to mutation yield (found in 52% of patients, 90% of pathogenic mutations). Previous studies reported *PKP2* mutations in 19% to 45% of ARVD/C patients and mutations in other desmosomal genes in 1% to 12%.[21,22,31,32] These differences can be due to various causes, such as the presence of founder mutations, use of different definitions for pathogenicity, regional differences in other genetic and nongenetic causes, and strictness in applying the TFC.[6,19] New genetic techniques, such as high-density genotyping array with haplotype sharing or exome sequencing, might elucidate new genes involved in ARVD/C.[33] This will improve the distinction between familial and sporadic ARVD/C cases and ameliorate risk stratification for relatives.

Our study suggests that UVs as defined here do not result in a more severe phenotype or earlier onset of ARVD/C. However, most UVs were found only in single patients; therefore, comparisons on the level of individual variants were not possible. Because the pathogenic influence of UVs is unknown, relatives were not tested for these variants. Consequently, it cannot be ruled out that, within families, carrying a specific UV is a risk factor for disease development.

Index patients were included only if they fulfilled the 2010 TFC. Consequently, this obviously affected population cannot be regarded as representative of the variable disease expression. Therefore, analysis of asymptomatic family members was crucial.

Family Members

The large majority (84%) of initially asymptomatic family members showing signs of ARVD/C carried desmosomal gene mutations (90% truncating, all *PKP2*). Overall, we found no differences between carriers of truncating and missense mutations. In 4 families with ≥2 members diagnosed with ARVD/C and in whom no mutations were identified, a genetic cause is highly suspected.

Negative T waves in V_1 to V_3 have always been considered the most sensitive ECG abnormality in ARVD/C.[17–20] However, in family members, we observed the new criterion of prolonged TAD more often and at younger age (Figure 3). In 4 of 7 family members <20 years of age with signs of ARVD/C, prolonged TAD was the only clinical abnormality observed. Longer periods of follow-up are needed to demonstrate the disease progression after prolonged TAD is found and whether it is a good predictive marker of arrhythmias and SCD.

Disease Penetrance

Because ARVD/C is a progressive disease with age-related penetrance, it seems counterintuitive that the percentage of family members showing any signs of ARVD/C was lower in those ≥50 years of age than in their younger counterparts. Because individuals ≥50 years of age are also at higher risk of coronary artery disease, those already seeing a cardiologist may not have been referred for family screening.

Cohorts of ARVD/C index patients universally demonstrate a male predominance.[18–21] Interestingly, among the asymptomatic family members in this study, women were affected more often than men. Apparently, men with ARVD/C experience arrhythmias and die suddenly at a younger age and thus are more likely to be the index patient. The underlying mechanism is not yet known, but strenuous exercise by men and the prevention of programmed cell death in cardiac myocytes in women due to estradiol are believed to play a role.[34]

At first examination, 43 of 282 asymptomatic family members (15%) already showed any sign of disease, including nonsustained VT in 5 patients and >500 premature ventricular complexes in 24 hours in 30 patients. Because relatives with these arrhythmias were asymptomatic, the age of onset is unknown. Besides, only 4 relatives (all mutation carriers) went on to develop VT during follow-up. Therefore, it was not possible to calculate the annual risk of arrhythmias or to identify predictive risk factors other than carrying a pathogenic mutation. Longer periods of follow-up of large series of relatives who initially have no signs of disease are needed to provide this insight. Large multicenter studies are required to achieve this.

Contrary to our study, all previous genotype-phenotype analyses in ARVD/C families separately addressed mutations in different desmosomal genes and involved only a few families. We found that 66 of 137 mutation-carrying family members (48%) showed some sign of ARVD/C, with ARVD/C diagnosis made according to 2010 TFC in 39%. No direct comparison with other studies can be made, because different sets of diagnostic criteria were used. However, similar numbers of affected relatives with *PKP2* mutations were reported by Dalal et al[35] and Syrris et al[20] (49% and 47%, respectively). Other family studies have also reported high percentages of relatives diagnosed with ARVD/C: 58% in *DSG2*, 75% in *DSC2*, and 54% in *DSP* mutation carriers. However, these studies comprised only 8, 2, and 4 families, respectively.[8,30,36]

Study Limitations

Sequence alterations were divided into pathogenic or UVs according to arbitrarily defined criteria. However, in the absence of any data on pathogenicity, conclusions regarding the utility of genetic testing are speculative.

All index patients were offered family screening, regardless of their mutational status. However, relatives of 93 of 149 fully genotyped index patients (62%) underwent both clinical and genetic screening. Although there is no such indication, this might potentially create a bias in the population of relatives studied. Not all relatives underwent all the diagnostic tests, as indicated in the Methods section. All family members included in this study underwent at least 2-dimensional echocardiography to screen for RV structural abnormalities. Additional magnetic resonance imaging was

performed in only a minority of cases, so minor abnormalities might have been missed. Late potentials were measured in 25% of relatives, but activation delay was measured by means of prolonged TAD in all relatives.

Thirty-six relatives of mutation-carrying index patients were not clinically evaluated because they appeared to be noncarriers; thus, they could not be included in this study. Consequently, of families with pathogenic mutations, more relatives with mutations were included than without mutations (119 versus 67).

Conclusions

In this large follow-up study in Dutch ARVD/C families, pathogenic desmosomal gene mutations were found in the majority of ARVD/C index patients (58%); these were mainly truncating *PKP2* mutations, with multiple mutations in 2% of cases. Performing multiplex ligation-dependent probe amplification also is important, because its mutational yield is comparable to the sequencing of *DSG2* or *DSC2*. Mutation carriers presented at a younger age than noncarriers; this age was even lower when multiple mutations were identified.

In total, 20% of initially asymptomatic relatives who underwent family screening showed some sign of ARVD/C (84% mutation carriers), mainly ECG abnormalities. Mutation-carrying relatives had an earlier onset of signs and symptoms, a markedly increased risk of arrhythmias, and a 6-fold increased risk of ARVD/C diagnosis. Familial cases were identified in 45% of the families screened. Prolonged TAD seems to be an early marker of RV abnormalities, especially in mutation-carrying relatives <20 years of age.

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Disclosures

None.

References

1. Marcus FI, Fontaine GH, Guiraudon G, Frank R, Laurenceau JL, Malergue C, Grosgogeat Y. Right ventricular dysplasia: report of 24 adult cases. *Circulation*. 1982;65:384–398.
2. Corrado D, Basso C, Thiene G, McKenna WJ, Davies MJ, Fontaliran F, Nava A, Silvestri F, Blomstrom-Lundqvist C, Wlodarska EK, Fontaine G, Camerini F. Spectrum of clinicopathologic manifestations of arrhythmogenic right ventricular cardiomyopathy/dysplasia: multicenter study. *J Am Coll Cardiol*. 1997;30:1512–1520.
3. Basso C, Thiene G, Corrado D, Angelini A, Nava A, Valente M. Arrhythmogenic right ventricular cardiomyopathy: dysplasia, dystrophy, or myocarditis? *Circulation*. 1996;94:983–991.
4. McKoy G, Protonotarios N, Crosby A, Tsatsopoulou A, Anastasakis A, Coonar A, Norman M, Baboonian C, Jeffery S, McKenna WJ. Identification of a deletion in plakoglobin in arrhythmogenic right ventricular cardiomyopathy with palmoplantar keratoderma and woolly hair (Naxos disease). *Lancet*. 2000;355:2119–2124.
5. Rampazzo A, Nava A, Malacrida S, Beffagna G, Bauce B, Rossi V, Zimbello R, Simionati B, Basso C, Thiene G, Towbin JA, Danieli GA. Mutation in human desmoplakin domain binding to plakoglobin causes a dominant form of arrhythmogenic right ventricular cardiomyopathy. *Am J Hum Genet*. 2002;71:1200–1206.
6. Gerull B, Heuser A, Wichter T, Paul M, Basson CT, McDermott DA, Lerman BB, Markowitz SM, Ellinor PT, MacRae CA, Peters S, Grossmann KS, Drenckhahn J, Michely B, Sasse-Klaassen S, Birchmeier W, Dietz R, Breithardt G, Schulze-Bahr E, Thierfelder L. Mutations in desmosomal protein plakophilin-2 are common in arrhythmogenic right ventricular cardiomyopathy. *Nat Genet*. 2004;36:1162–1164.
7. Pilichou K, Nava A, Basso C, Beffagna G, Bauce B, Lorenzon A, Frigo G, Vettori A, Valente M, Towbin J, Thiene G, Danieli GA, Rampazzo A. Mutations in desmoglein-2 gene are associated with arrhythmogenic right ventricular cardiomyopathy. *Circulation*. 2006;113:1171–1179.
8. Syrris P, Ward D, Evans A, Asimaki A, Gandjbakhch E, Sen-Chowdhry S, McKenna WJ. Arrhythmogenic right ventricular dysplasia/cardiomyopathy associated with mutations in desmosomal gene desmocollin-2. *Am J Hum Genet*. 2006;79:978–984.
9. Asimaki A, Syrris P, Wichter T, Matthias P, Saffitz JE, McKenna WJ. A novel dominant mutation in plakoglobin causes arrhythmogenic right ventricular cardiomyopathy. *Am J Hum Genet*. 2007;81:964–973.
10. Asimaki A, Tandri H, Huang H, Halushka MK, Gautam S, Basso C, Thiene G, Tsatsopoulou A, Protonotarios N, McKenna WJ, Calkins H, Saffitz JE. A new diagnostic test for arrhythmogenic right ventricular cardiomyopathy. *N Engl J Med*. 2009;360:1075–1084.
11. Basso C, Czarnowska E, Della Barbera M, Bauce B, Beffagna G, Wlodarska EK, Pilichou K, Ramondo A, Lorenzon A, Wozniek O, Corrado D, Daliento L, Danieli GA, Valente M, Nava A, Thiene G, Rampazzo A. Ultrastructural evidence of intercalated disc remodelling in arrhythmogenic right ventricular cardiomyopathy: an electron microscopy investigation on endomyocardial biopsies. *Eur Heart J*. 2006;27:1847–1854.
12. Oxford EM, Musa H, Maass K, Coombs W, Taffet SM, Delmar M. Connexin43 remodeling caused by inhibition of Plakophilin-2 expression in cardiac cells. *Circ Res*. 2007;101:703–711.
13. Garcia-Gras E, Lombardi R, Giocondo MJ, Willerson JT, Schneider MD, Khoury DS, Marian AJ. Suppression of canonical Wnt/beta-catenin signaling by nuclear plakoglobin recapitulates phenotype of arrhythmogenic right ventricular cardiomyopathy. *J Clin Invest*. 2006;116:2012–2021.
14. De Bakker JM, van Capelle FJ, Janse MJ, Tasseron S, Vermeulen JT, de Jonge N, Lahpor JR. Slow conduction in the infarcted human heart: "zigzag" course of activation. *Circulation*. 1993;88:915–926.
15. Cabo C, Pertsov AM, Baxter WT, Davidenko JM, Gray RA, Jalife J. Wave-front curvature as a cause of slow conduction and block in isolated cardiac muscle. *Circ Res*. 1994;75:1014–1028.
16. Fast VG, Kleber AG. Role of wavefront curvature in propagation of cardiac impulse. *Cardiovasc Res*. 1997;33:258–271.
17. Hamid MS, Norman M, Quraishi A, Firoozi S, Thaman R, Gimeno JR, Sachdev B, Rowland E, Elliott PM, McKenna WJ. Prospective evaluation of relatives for familial arrhythmogenic right ventricular cardiomyopathy/dysplasia reveals a need to broaden diagnostic criteria. *J Am Coll Cardiol*. 2002;40:1445–1450.
18. Dalal D, Molin LH, Piccini J, Tichnell C, James C, Bomma C, Prakasa K, Towbin JA, Marcus FI, Spevak PJ, Bluemke DA, Abraham T, Russell SD, Calkins H, Judge DP. Clinical features of arrhythmogenic right ventricular dysplasia/cardiomyopathy associated with mutations in plakophilin-2. *Circulation*. 2006;113:1641–1649.
19. Van Tintelen JP, Entius MM, Bhuiyan ZA, Jongbloed R, Wiesfeld AC, Wilde AA, van der Smagt J, Boven LG, Mannens MM, van Langen IM, Hofstra RM, Otterspoor LC, Doevendans PA, Rodriguez LM, van Gelder IC, Hauer RN. Plakophilin-2 mutations are the major determinant of familial arrhythmogenic right ventricular dysplasia/cardiomyopathy. *Circulation*. 2006;113:1650–1658.
20. Syrris P, Ward D, Asimaki A, Sen-Chowdhry S, Ebrahim HY, Evans A, Hitomi N, Norman M, Pantazis A, Shaw AL, Elliott PM, McKenna WJ. Clinical expression of plakophilin-2 mutations in familial arrhythmogenic right ventricular cardiomyopathy. *Circulation*. 2006;113:356–364.
21. Den Haan AD, Tan BY, Zikusoka MN, Llado LI, Jain R, Daly A, Tichnell C, James C, Amat-Alarcon N, Abraham T, Russell SD, Bluemke DA, Calkins H, Dalal D, Judge DP. Comprehensive desmosome mutation analysis in North Americans with arrhythmogenic right ventricular dysplasia/cardiomyopathy. *Circ Cardiovasc Genet*. 2009;2:428–435.

22. Bauce B, Nava A, Beffagna G, Basso C, Lorenzon A, Smaniotto G, De Bortoli M, Rigato I, Mazzotti E, Steriotis A, Marra MP, Towbin JA, Thiene G, Danieli GA, Rampazzo A. Multiple mutations in desmosomal proteins encoding genes in arrhythmogenic right ventricular cardiomyopathy/dysplasia. *Heart Rhythm*. 2010;7:22–29.
23. Bhuiyan ZA, Jongbloed JD, van der Smagt J, Lombardi PM, Wiesfeld AC, Nelen M, Schouten M, Jongbloed R, Cox MGPJ, van Wolferen M, Rodriguez LM, van Gelder IC, Bikker H, Suurmeijer AJ, van den Berg MP, Mannens MM, Hauer RN, Wilde AA, van Tintelen JP. Desmoglein-2 and desmocollin-2 mutations in Dutch arrhythmogenic right ventricular dysplasia/cardiomyopathy patients: results from a multicenter study. *Circ Cardiovasc Genet*. 2009;2:418–427.
24. Fressart V, Duthoit G, Donal E, Probst V, Deharo JC, Chevalier P, Klug D, Dubourg O, Delacretaz E, Cosnay P, Scanu P, Extramiana F, Keller D, Hidden-Lucet F, Simon F, Bessirard V, Roux-Buisson N, Hebert JL, Azarine A, Casset-Senon D, Rouzet F, Lecarpentier Y, Fontaine G, Coirault C, Frank R, Hainque B, Charron P. Desmosomal gene analysis in arrhythmogenic right ventricular dysplasia/cardiomyopathy: spectrum of mutations and clinical impact in practice. *Europace*. 2010;12:861–868.
25. Marcus FI, McKenna WJ, Sherrill D, Basso C, Bauce B, Bluemke DA, Calkins H, Corrado D, Cox MGPJ, Daubert JP, Fontaine G, Gear K, Hauer R, Nava A, Picard MH, Protonotarios N, Saffitz JE, Sanborn DM, Steinberg JS, Tandri H, Thiene G, Towbin JA, Tsatsopoulou A, Wichter T, Zareba W. Diagnosis of arrhythmogenic right ventricular cardiomyopathy/dysplasia: proposed modification of the task force criteria. *Circulation*. 2010;121:1533–1541.
26. Fontaine G, Umemura J, Di Donna P, Tsezana R, Cannat JJ, Frank R. Duration of QRS complexes in arrhythmogenic right ventricular dysplasia: a new non-invasive diagnostic marker. *Ann Cardiol Angiol*. 1993;42:399–405.
27. Cox MGPJ, Nelen MR, Wilde AAM, Wiesfeld AC, Van der Smagt JJ, Loh KP, Cramer MJ, Doevendans PA, Van Tintelen JP, De Bakker JMT, Hauer RNW. Activation delay and VT parameters in arrhythmogenic right ventricular dysplasia/cardiomyopathy: towards improvement of diagnostic ECG criteria. *J Cardiovasc Electrophysiol*. 2008;19:775–781.
28. Ramensky V, Bork P, Sunyaev S. Human non-synonymous SNPs: server and survey. *Nucleic Acids Res*. 2002;30:3894–3900.
29. Ng PC, Henikoff S. SIFT: Predicting amino acid changes that affect protein function. *Nucleic Acids Res*. 2003;31:3812–3814.
30. Syrris P, Ward D, Asimaki A, Evans A, Sen-Chowdhry S, Hughes SE, McKenna WJ. Desmoglein-2 mutations in arrhythmogenic right ventricular cardiomyopathy: a genotype-phenotype characterization of familial disease. *Eur Heart J*. 2007;28:581–588.
31. Van der Zwaag PA, Jongbloed JD, van den Berg MP, van der Smagt JJ, Jongbloed R, Bikker H, Hofstra RM, van Tintelen JP. A genetic variants database for arrhythmogenic right ventricular dysplasia/cardiomyopathy. *Hum Mutat*. 2009;30:1278–1283.
32. Xu T, Yang Z, Vatta M, Rampazzo A, Beffagna G, Pilichou K, Scherer SE, Saffitz J, Kravitz J, Zareba W, Danieli GA, Lorenzon A, Nava A, Bauce B, Thiene G, Basso C, Calkins H, Gear K, Marcus F, Towbin JA; Multidisciplinary Study of Right Ventricular Dysplasia Investigators. Compound and digenic heterozygosity contributes to arrhythmogenic right ventricular cardiomyopathy. *J Am Coll Cardiol*. 2010;55:587–597.
33. Van der Zwaag PA, Van Tintelen JP, Gerbens F, Jongbloed JDH, Boven LG, Van der Smagt JJ, Van der Roest WP, Van Langen IM, Bikker H, Hauer RNW, Van den Berg MP, Hofstra RMW, Te Meerman GJ. Haplotype sharing test maps genes for familial cardiomyopathies. *Clin Genet*. 2011;79:459–467.
34. Pelzer T, Schumann M, Neumann M, deJager T, Stimpel M, Serfling E, Neyses L. 17Beta-estradiol prevents programmed cell death in cardiac myocytes. *Biochem Biophys Res Commun*. 2000;268:192–200.
35. Dalal D, James C, Devanagondi R, Tichnell C, Tucker A, Prakasa K, Spevak PJ, Bluemke DA, Abraham T, Russell SD, Calkins H, Judge DP. Penetrance of mutations in plakophilin-2 among families with arrhythmogenic right ventricular dysplasia/cardiomyopathy. *J Am Coll Cardiol*. 2006;48:1416–1424.
36. Bauce B, Basso C, Rampazzo A, Beffagna G, Daliento L, Frigo G, Malacrida S, Settimo L, Danieli G, Thiene G, Nava A. Clinical profile of four families with arrhythmogenic right ventricular cardiomyopathy caused by dominant desmoplakin mutations. *Eur Heart J*. 2005;26:1666–1675.

CLINICAL PERSPECTIVE

Arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C) usually shows an autosomal dominant inheritance pattern, with incomplete penetrance and variable clinical expression. Classically, index patients present between the second and fourth decades of life with right ventricular tachycardia. However, sudden death can occur at adolescence, whereas mutation carriers may remain without signs and symptoms until old age. Previous genotype-phenotype studies involved mainly overt index patients. Data on mainly asymptomatic relatives were scarce. To gain insight into the full spectrum of the disease, 149 ARVD/C index patients and 302 of their relatives were genotypically and phenotypically characterized. DNA analysis comprised sequencing of the desmosomal genes *PKP2, DSC2, DSG2, DSP,* and *JUP* and multiplex ligation-dependent probe amplification to identify large deletions in *PKP2*. Pathogenic mutations were identified in 87 of 149 ARVD/C index patients (58%), mainly truncating *PKP2* mutations with multiple mutations in 2% of cases. Performing multiplex ligation-dependent probe amplification also appeared to be important; its 2% mutational yield was comparable to the sequencing of *DSG2* or *DSC2*. Identification of mutations in index patients had major consequences for the concurrent relatives. Of the 57 of 282 initially asymptomatic relatives (20%) who showed any sign of ARVD/C, 84% carried a mutation. These 48 mutation carriers had earlier onset of disease signs and symptoms than noncarriers but also a markedly increased risk of arrhythmias and 6-fold risk of ARVD/C diagnosis. Familial cases were identified in 45% of families screened. Prolonged terminal activation duration seemed to be an early marker of ARVD/C; it was observed more often than negative T waves in V_1 to V_3, especially in mutation-carrying relatives <20 years of age.

SUPPLEMENTAL MATERIAL

Supplemental Table 1:

Follow-up Frequencies of Index-patients and Relatives

	Index-Patients (n=147)		Relatives (n=302)	
	mean±SD	median (range)	mean±SD	median (range)
Intervals (years) between				
Visits	0.5±0.3	0.3 (0.2-1.2)	1.1±0.6	1 (0.3-5.0)
Echocardiography	3.6±2.3	2.8 (1-9)	1.7±1.5	1 (1-12)
Exercise Tests	3.1±2.0	2.5 (0.8-9)	2.6±2.1	2 (1-10)
24h Holter	5.1±4.7	2.5 (0.8-16)	2.6±2.0	2 (1-10)

Supplemental Table 2: Unclassified Variants

				PolyPhen (PSIC)*	SIFT†	Index-patients n
Unclassified Variants						
PKP2	c.76G>A	p.Asp26Asn	Missense	+ (1.593)	0.15	2
	c.174G>T	Glu58Asp	Missense	- (1.248)	0.42	1
	c.759C>T	p.=	Silent			1
	c.1378G>A	p.Asp460Asn	Missense	- (0.0)	0.45	1
DSC2	c.1807A>C	p.Ile603Leu	Missense	- (1.157)	0.20	1
	c.1914G>T	p.Gln638His	Missense	+ (1.710)	0.01‡	1
	c.2194T>G	p.Leu732Val	Missense	- (0.197)	0.15	2
	c.2393G>A	p.Arg798Gln	Missense	- (0.716)	0.00‡	1
	c.2686_2687dupGA	p.Ala897LysfsX4	Frameshift			8
DSG2	c.445G>T	p.Val149Phe	Missense	+ (1.747)	0.00‡	1
	c.473T>G	p.Val158Gly	Missense	++ (2.470)	0.00‡	2
	c.783T>A	p.=	Silent			1
	c.889G>A	p.Asp297Asn	Missense	+ (1.951)	0.00‡	1
	c.1003A>G	p.Thr335Ala	Missense	+ (1.559)	0.00‡	3
	c.1072G>A	p.Ala358Thr	Missense	+ (1.760)	0.00‡	1
	c.1174G>A	p.Val392Ile	Missense	- (0.813)	0.14	1
	c.1303G>A	p.Asp435Asn	Missense	- (1.459)	0.19	1
	c.1480G>A	p.Asp494Asn	Missense	+ (1.790)	0.00‡	1
	c.2137G>A	p.Glu713Lys	Missense	+ (1.632)	0.09	1
	c.2194T>G	p.Phe732Val	Missense	- (0.217)	0.56	1
	c.2623A>G	p.Met875Val	Missense	- (0.682)	0.65	1
	c.2759T>G	p.Val920Gly	Missense	+ (1.936)	0.21	1
DSP	c.105G>A	p.=	Silent			1
	c.269A>G	p.Gln90Arg	Missense	- (1.432)	0.00‡	1
	c.1696G>A	p.Ala566Thr	Missense	- (1.227)	0.00‡	1
	c.2346C>T	p.=	Silent			1
	c.4372C>G	p.Arg1458Gly	Missense	+ (1.727)	0.03‡	1
	c.4775A>G	p.Lys1592Arg	Missense	- (1.047)	0.00‡	1
	c.5218G>A	p.Glu1740Lys	Missense	- (1.267)	0.00‡	2
	c.5498A>T	p.Glu1833Val	Missense	+ (1.942)	0.00‡	1
	c.6449C>G	p.Ala2150Gly	Missense	- (0.986)	0.08	1
JUP	c.297G>A	p.=	Silent			1
	c.1359G>T	p.Glu453Asp	Missense	+ (1.616)	0.00‡	1
	c.1563A>G	p.=	Silent			2
	c.1942G>A	p.Val648Ile	Missense	- (0.417)	1.00	1

* PolyPhen prediction: ++ probably damaging; + possibly damaging; - benign; † SIFT prediction: Amino acids with scores < 0.05 are predicted to be deleterious. For previous reports on pathogeneity: see reference 31; ‡This substitution may have been predicted to affect function just because the sequences used were not diverse enough. There is low confidence in this prediction; § The 4 splice-prediction programs used (SpliceSiteFinder, MaxEntScan, NNSPLICE and GeneSplicer) indicated disruption the splice donor site of DSC2 exon 7.

Supplemental Table 3: Clinical Characteristics of ARVD/C Probands with 2 Pathogenic Mutations

Gender	Mutation 1	Mutation 2	Age First VT (years)	Depolarization Abnormalities			Repolarization Abnormalities:	Arrhythmias	Structural Abnormalities
				ε	LP	TAD	Negative T waves V1-3	LBBB VT	
V	PKP2: 917-918delCC	DSC2: 2587G>A	14				++	+	++
V	PKP2: 2489+4A>C	DSP: 1982A>T	20	++		+	++	++	++
V	DSC2: 608G>A homozygous *		17	++		+	++	+	++

ε = epsilon waves, LP= late potentials, TAD = prolonged terminal activation duration; LBBB VT: ventriculat tachycardia with left bundle branch block morphology
+ minor criterion, ++ major criterion; * Parents were first degree cousins

Supplemental Table 4: Combinations of Pathogenic Mutations and/or Unclassified Variants

	PKP2	DSC2	DSG2	DSP	JUP
2 Mutations (+UV)					
	917-918delCC	***2587G>A***			
	2489+4A>C			***1982A>T***	
		608G>A homozygous			
1 Mutation + UV					
	deletion exon 8	2686_2687dupGA			
	deletion exon 1-14		*1072G>A*		
	148_151delAC AG	2686_2687dupGA			
	235C>T	2686_2687dupGA			
	235C>T		*1003A>G*		
	235C>T		*1174G>A*		
	397C>T		*1480G>A*		
	397C>T		*2623A>G*		
	397C>T		*2759T>G*		
	917_918delCC		*473T>G*		
	1211-1212insT	*2194T>G*			
	1211-1212insT	2686_2687dupGA			
	1211-1212insT			*269A>G*	
	1211-1212insT			*5218G>A*	
	1848C>A				*1942G>A*
	2146-1G>C		*1696G>A*		
	2386T>C		*1003A>G*		
	2386T>C				*297G>A*
	2421C>A	2686_2687dupGA			
	2489+1G>A		*1303G>A*		
	2489+4A>C			*5218G>A*	
	2489+1G>A				*1563A>G*
	2489+4A>C				
	2544G>A	2686_2687dupGA			
		943-1G>A		*6449C>G*	
			874C>T	*2346C>T*	
			137G>A + *473T>G*		
			137G>A + *473T>G*		
				3337C>T	*4372C>G*
2 UVs		*2194T>G*	*1174G>A*		
			445G>T		*1359G>T*

UV= unclassified variant; In **bold**: (most likely) pathogenic mutations; In *italics*: missense and silent variants.

Supplemental Table 5: Clinical Characteristics of ARVD/C Index-Patients Related to Pathogenic Mutations and Unclassified Variants

	Mutations and Unclassified Variants (UVs)				
	0	**uv**	**1**	**1+uv**	**2**
n	40	20	55	29	3
% male	81	76	78	66	0
age onset (years±SD)	41±14	38±12	35±12	34±15	17±3
	n (%)	n (%)	n (%)	n (%)	n (%)
epsilon wave †	7 (24)	1 (6)	10 (19)	2 (7)	2 (67)
late potentials ‡	14 (47)	8 (67)	11 (31)	11 (52)	0
prolonged TAD†	24 (83)	10 (56)	30 (57)	12 (52)	2 (67)
negative T waves V1-3 †	18 (62)	11 (61)	45 (85)	21 (72)	3 (100)
negative T waves V1-2 †	1 (3)	2 (11)	4 (8)	1 (3)	0
negative T waves V4-6 †	1 (3)	2 (11)	1 (2)	1 (3)	0
LBBB VT superior axis	17 (43)	11 (55)	24 (44)	14 (48)	1 (33)
LBBB VT	34 (85)	18 (90)	48 (87)	26 (90)	3 (100)
PVCs >500 per 24h	9 (23)	4 (20)	14 (25)	5 (17)	1 (33)
structural major TFC	17 (43)	14 (70)	32 (58)	20 (69)	3 (100)
structural minor TFC	8 (20)	2 (10)	12 (22)	3 (10)	0

† Of 125 probands, ECGs while off drugs were available; ‡ In 81 patients late potentials were measured: 69 by SAECG, 12 by mapping during EPS; TAD: terminal activation duration, LBBB VT: ventricular tachycardia with left bundle branch block morphology, PVCs: premature ventricular complexes, TFC: Task Force Criteria.