Arrhythmogenic Right Ventricular Cardiomyopathy Type 5 Is a Fully Penetrant, Lethal Arrhythmic Disorder Caused by a Missense Mutation in the *TMEM43* Gene

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Autosomal-dominant arrhythmogenic right ventricular cardiomyopathy/dysplasia (ARVC/D) causes sudden cardiac death and is characterized by clinical and genetic heterogeneity. Fifteen unrelated ARVC families with a disease-associated haplotype on chromosome 3p (*ARVD5*) were ascertained from a genetically isolated population. Identification of key recombination events reduced the disease region to a 2.36 Mb interval containing 20 annotated genes. Bidirectional resequencing showed one rare variant in *transmembrane protein 43* (*TMEM43* 1073C→T, S358L), was carried on all recombinant *ARVD5* ancestral haplotypes from affected subjects and not found in population controls. The mutation occurs in a highly conserved transmembrane domain of TMEM43 and is predicted to be deleterious. Clinical outcomes in 257 affected and 151 unaffected subjects were compared, and penetrance was determined. We concluded that ARVC at locus *ARVD5* is a lethal, fully penetrant, sex-influenced morbid disorder. Median life expectancy was 41 years in affected males compared to 71 years in affected females (relative risk 6.8, 95% CI 1.3–10.9). Heart failure was a late manifestation in survivors. Although little is known about the function of the *TMEM43* gene, it contains a response element for PPARγ (an adipogenic transcription factor), which may explain the fibrofatty replacement of the myocardium, a characteristic pathological finding in ARVC.

Introduction

Arrhythmogenic right ventricular cardiomyopathy/dysplasia (ARVC/D [MIM 107970]) is an inherited disorder, often involving both ventricles, and is characterized by ventricular tachycardia (VT), heart failure, sudden cardiac death (SCD), and fibrofatty replacement of cardiomyocytes.[1] It is usually inherited as an autosomal-dominant disorder, although recessive forms exist.[2] To date, eleven genetic loci have been mapped and seven genes have been identified.[3–7] Five genes code for desmosomal proteins (desmoplakin [MIM 125647], plakophilin-2 [MIM 602861], desmoglein-2 [MIM 125671], desmocollin-2 [MIM 125645], and plakoglobin [MIM 173325]) that are predicted to succumb to mechanical stress.[5,7] The remaining two genes are *cardiac ryanadine receptor 2* (*RYR2* [MIM 180902])[8] and *transforming growth factor beta-3* (*TGFβ3* [MIM 190230]).[9]

Clinical diagnosis of ARVC is difficult because it relies on physiological and pathological testing for the presence of right ventricular structural and functional anomalies, fibrofatty replacement of the ventricular myocardium, premature ventricular contractions (PVCs) on Holter monitor, extended QRS, epsilon waves and T wave inversion on 12 lead ECG, and late potentials on signal-averaged ECG (SAECG).[10] Phenotypic variation of ARVC, in both presen-

tation and clinical course, has led to a modification of the McKenna diagnostic criteria[10] to facilitate the identification of relatives at risk in ARVC families.[11] ARVC can have both adverse (sudden death and heart failure)[12] and favorable outcomes,[13] which may be due to genetic heterogeneity, although clearly mutations at different loci do not explain intrafamilial variation[14] or the sex influence toward greater clinical severity in males.[15] There are no known studies to date that assess clinical features in mutation-negative subjects born at a priori 50% pedigree risk to determine mutation-specific cardiac features: Most studies assess small families to determine cardiac features and gene-specific (not mutation-specific) penetrance.[16] Ultimately, the determination of the penetrance of mutation-specific clinical manifestations of ARVC will provide accurate, mutation-specific clinical information for appropriate genetic counseling and treatment options.
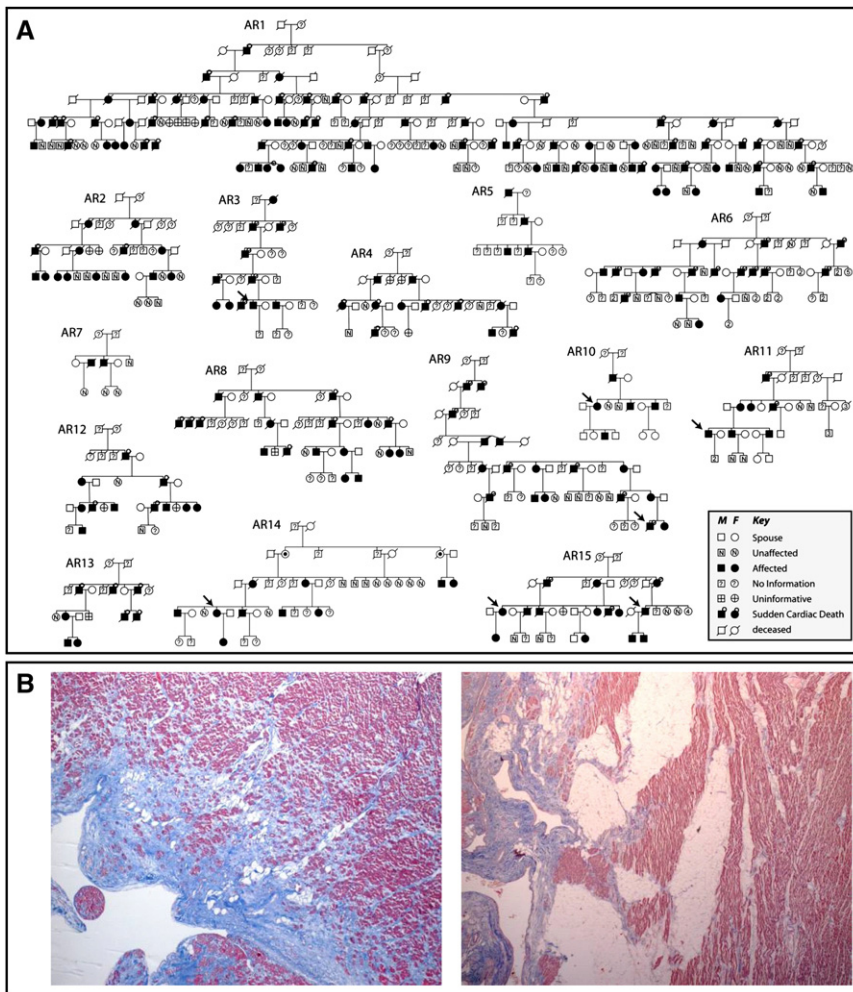
The *ARVD5* locus (MIM 604400) on 3p was mapped in an extended eight generation family from the genetically isolated population of the island of Newfoundland, Canada.[17] This family was first identified in the 1980s,[18] and at least one family relative participated in right ventricular disconnection studies as a possible treatment for ARVC.[19] We have identified 14 additional Newfoundland-ancestral families that share the *ARVD5* haplotype and have previously shown that the use of prophylactic treatment with

with the Human Investigation Committee requirements of the Eastern Health Corporation of St. John's, Newfoundland, Canada (study number 00-176).

Fine Mapping of the *ARVD5* Locus

Mapping the *ARVD5* locus in family AR1 (Figure 1A) previously defined a 9.3 cM disease region between markers *D3S3610* and *D3S3659*.[17] We genotyped 18 polymorphic microsatellite markers on clinically affected subjects across all families to identify a disease-associated haplotype at 3p25 that was shared among families from this genetic isolate and presumed to be ancestral. We identified key recombination events on the *ARVD5* ancestral haplotype and used recombinations seen in two or more families to narrow the region.

Screening Candidate *ARVD5* Genes

A mutation-screening panel was established that comprised seven genomic DNA samples from four clinically affected subjects from three families (AR1, AR8, and AR15; Figure 1A) and three spouses (controls). All coding and noncoding exons and intron-exon boundaries of positional candidate genes for *ARVD5* were sequenced. All sequences were amplified by polymerase-chain reaction (PCR) assay from genomic DNA in 25 μl reaction volume. Primer sequences are available from the authors on request. The PCR products were purified with 50% sephacryl (Amersham Biosciences) and MultiScreen HTS filter plates (Millipore Corporation). Purified PCR products were cycle sequenced in both forward and reverse directions with the use of BigDye Terminator V3.1 cycle sequencing kit on an automated ABI 3700 DNA analyzer (Applied Biosystems). Sequencing electropherograms were inspected manually and analyzed with Mutation Surveyor software (Transition Technologies). Sequencing variants found exclusively in clinically affected subjects on the mutation-screening panel were experimentally determined to reside on the *ARVD5* haplotype by segregation analysis in family AR14 (Figure 1A). The allele frequencies of the *ARVD5* sequencing

implantable cardioverter defibrillator (ICD) therapy in relatives at risk of ARVC greatly improved survival.[20] We report here that we have identified the *ARVD5* disease gene and determined the mutation-specific penetrance of its major clinical manifestations.

Subjects and Methods

Study Population

Fifteen of 150 unrelated families were referred either to the Newfoundland Provincial Medical Genetics Program or the Newfoundland Labrador genetics cardiomyopathy clinic because of a family history of cardiomyopathy and sudden death. These families were determined to have ARVC on the basis of clinical testing with established criteria,[10] postmortem pathology, and an autosomal-dominant pattern of inheritance (Figures 1A and 1B). These families are characterized by deep genealogies, a Newfoundland ancestry, and a disease-associated haplotype originally identified in family AR1 used to map *ARVD5* (Figure 1A).

A total of 496 subjects born at a priori 50% risk (clinically affected and their first-degree relatives) were available for study across the 15 families. Subjects were determined to be clinically affected if they fulfilled the ARVC modified criteria[11] (Figure 2). Blood samples from 295 subjects born at a priori 50% risk were collected, and informed consent was obtained in compliance

Figure 2 workflow

15 ARVD5-linked families

Total subjects born at a priori 50% risk, no DNA
n=201
Male = 135
Female=66

Total subjects born at a priori 50% risk, with DNA
n=295
Male = 135
Female=160

Clinically Affected
n=113
Male=90
Female=23

Disease Status Unknown
n=88

Clinically Affected
n=83
Male=40
Female=43

Clinically Unaffected
n=212
Male=95
Female=117

Non-Carriers
n=0
Male=0
Female=0

Mutation Carriers
n=83
Male=40
Female=43

Mutation Carriers
n=61
Male=18
Female=43

Non-Carriers
n=151
Male=77
Female=74

Mutation carriers
n=144
Male=58
Female=86

AFFECTED SAMPLE
n=257
Male=148
Female=109

UNAFFECTED SAMPLE
n=151
Male=77
Female=74

Disease status criteria pre mutation testing:

Clinically Affected: (1) Sudden cardiac death consistent with ARVC under 50 years of age
(at least one of) (2) VT or VF under 50 years of age + treatment with drugs or ICD or cardioversion
(3) Obligate carrier status (has a clinically affected parent and a child)

Clinically unaffected: (1) Presented for screening based on family history

Disease status criteria post mutation testing:

Affected: (1) Clinically affected
(at least one of) (2) Mutation positive

Unaffected: (1) Mutation negative

Unknown: (1) Unavailable for analysis

Figure 2. Workflow and Mutation Status of Subjects Born at a Priori 50% Risk of ARVC

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sites were predicted with the online tools via the ExPASy web site.[26] The effects of amino acid substitutions on protein function were predicted.[27–31] Information on specific analyses is available upon request.

Clinical Assessment of Affected versus Unaffected Subjects

Clinical data were prospectively collected over 11 years. This involved annual visits by subjects to the genetics cardiomyopathy clinic in which 12 lead ECGs, Holter monitors, MRIs, signal-averaged ECGs, and echocardiograms were done. All cardiac anomalies were noted after clinical testing. Clinical data were also obtained retrospectively from medical records including "at-risk" relatives not seen in clinic and autopsy results. Subjects were categorized as affected, unaffected, or unknown (Figure 2). Only subjects from well-ascertained sibships (in which disease status was known in ≥50% of siblings) were included in this study.

Anomalies on 12 lead ECGs were determined by two physicians blind to disease status. Left ventricular enlargement (LVE) was defined as ≥2 standard deviations (SDs) above a predicted mean: left ventricular end diastolic diameter (LVEDD) >112% (>2 SD).[32] Late potentials on SAECG were classified on the basis of recognized criteria: QRS (filtered QRS duration) >114 ms, LAS (low-amplitude signals) >38 ms, and RMS (root-mean-square voltage of the terminal 40 ms of filtered QRS) <20 ms.[33] The presence of arrhythmias in the form of PVCs was determined from Holter monitor analysis. Heart failure was classified according to the New York Heart Association (NYHA) functional classification.[34] Subjects were identified with heart failure (NYHA categories 1–4) if they presented prospectively in the study with heart failure or it was documented in a medical record. Date of death was confirmed via autopsy records and archival records.

Penetrance is defined as the proportion of subjects who have a specified genotype known to cause a disease and who have any signs or symptoms of the disease.[35] To determine the disease penetrance of ARVC at ARVD5, we assessed a subset of disease features based on the ARVC modified diagnostic criteria[11] from the first available clinical test result to show an anomaly. We could then assess which clinical features truly segregated with affection status in this population. A Kaplan Meier analysis was then done with a subset of subjects who were alive at the start of the prospective study (1996), who had an available medical record, and who were either mutation positive or an obligate carrier (60 males and 77 females). On the basis of the analysis of prevalent disease features, a male subject was considered "penetrant" at the age when any one of

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variants were determined with Newfoundland-population-based controls obtained through random phone dialing, as part of a large colorectal cancer study.[21] Newfoundland is a known genetic isolate where 98% of its residents are of English and Irish descent.[22,23] We analyzed key recombinant families (AR2 and AR10) to determine which rare variants (<1% of the alleles screened) were retained on recombinant ancestral haplotypes in clinically affected subjects.

Expression of *TMEM43* in Myocardium and Lymphocytes of Clinically Affected Subjects

Total RNA was extracted from Epstein Barr virus (EBV)-transformed B lymphocytes from two affected subjects and one unaffected control and also extracted from cardiac tissue from one affected subject and an unrelated control with Trizol (Invitrogen); this was followed by DNase1 treatment (Ambion). Complementary synthesis was performed and analyzed by both size fractionation and direct sequencing with overlapping primers designed to cover the complete coding sequence of TMEM43.

Bioinformatic Analysis

Conservation of the TMEM43 protein across species was determined with ClustalW and Weblogo.[24–26] Potential protein localization, function, structure, and posttranslational modification

Marker	D3S1259	D3S3610	D3S2403	D3S1516	D3S3608	D3S2385	D3S3602	D3S1585	D3S1554	D3S3595	D3S3613	D3S3473	D3S2338	D3S4547	D3S3510	D3S1293	D3S3038	D3S3659
Build 36.1 (Mb)	12.07	12.98	13.15	13.63	13.68	13.85	13.90	13.92	14.34	14.62	15.34	16.50	16.82	17.93	19.07	21.90	21.92	22.91
AR1	3	246	251	347	165	146	117	118	129	265	193	214	185	233	285	8	4	4
AR2	3	246	251	347	165	146	117	118	129	265	201	218	185	229	283	12	5	5
AR3	3	246	251	347	165	146	117	118	129	265	193	214	185	233	285	12	5	5
AR4	3	256	251	347	165	146	117	118	129	265	193	214	185	233	285	7	4	4
AR5	nd	246	251	nd	165	146	117	118	129	265	193	nd	nd	233	nd	nd	nd	nd
AR6	12	246	251	347	165	146	117	118	129	265	193	214	185	233	285	8	4	4
AR7	3	246	251	347	165	146	117	118	129	265	193	214	185	233	285	8	4	4
AR8	3	256	251	347	165	146	117	118	129	265	193	214	185	233	285	8	4	4
AR9	3	246	251	347	165	146	117	118	129	265	193	214	185	233	285	8	4	4
AR10	3	246	251	347	165	146	117	118	129	265	195	214	185	229	283	8	5	4
AR11	3	246	251	347	165	146	117	118	129	265	193	214	185	233	285	8	4	4
AR12	3	246	251	347	165	146	117	118	129	265	193	214	185	233	285	8	4	4
AR13	3	246	251	347	165	146	117	118	129	265	193	214	185	233	285	13	5	4
AR14	nd	nd	251	347	165	146	117	118	129	265	193	214	185	233	285	nd	nd	nd
AR15	7	246	251	351	165	146	117	118	129	265	193	214	187	229	283	7	nd	5
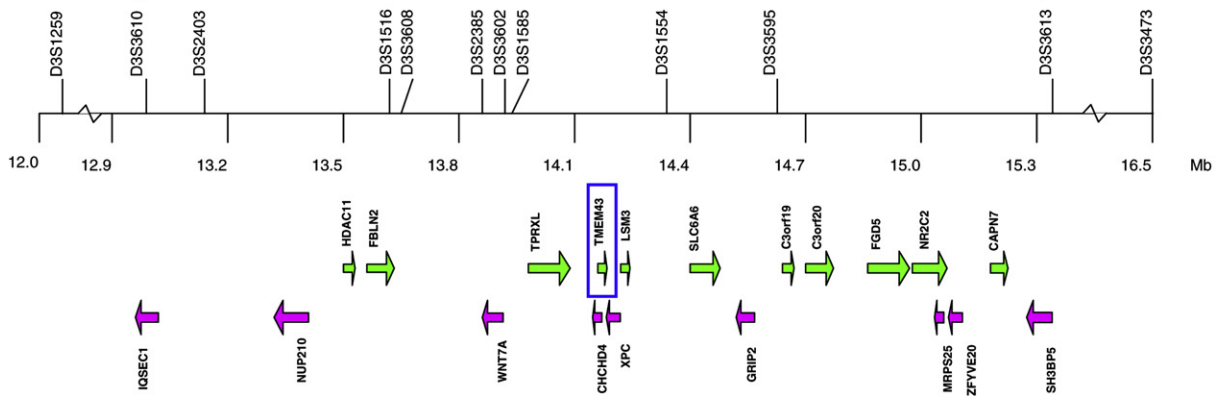
Figure 3. Physical Map of the ARVD5 Critical Region

(A) Summary recombinant *ARVD5* haplotypes identified across the 15 ARVC families with microsatellite markers (alleles are either numbered [1–9] or given in base pairs).

(B) The physical map of the *ARVD5* critical region. Physical distances were captured from the March 2006 freeze of the UCSC Genome Browser. Arrows show the direction of transcription of each annotated gene.

the following clinical events occurred: (1) SCD, (2) VT on clinical testing, (3) heart failure, (4) >200 PVCs in a 24 hr period, (5) LVE >2 SD, (6) QRS > 110 ms on 12 lead ECG, or (7) any late potential on SAECG.[11] Female subjects were considered penetrant for all these features apart from QRS >110 ms on 12 lead ECG and RMS or LAS on SAECG. We also determined the penetrance of two major morbid outcomes of the disease, death and heart failure, using both the prospective and retrospective data set (affected subjects n = 257, 148 males, 109 females; unaffected subjects, n = 151, 77 males, 74 females). For the heart-failure analysis, only those from this group with an available medical record were used.

Cardiac tests were not available in all subjects at a priori 50% risk, mainly because of SCD as a presenting feature for many family members. For example, of 114 affected subjects who did not have an available echocardiogram, 86 were deceased (75%), whereas only two were dead in 50 unaffected subjects with no echocardiogram. Testing is in progress in this latter group.

Statistical Analysis

Comparisons between affected versus unaffected subjects were calculated by the Kaplan Meier product limit method with censoring occurring at the time of ICD therapy, heart transplantation, or last follow-up (defined as the age at the last clinic visit). Relative risk was calculated with Cox's Regression model. A p value of <0.05 was considered significant (SPSS software, version 14, Chicago, IL).

Results

Fine Mapping and Candidate Gene Screening

Comparison of the *ARVD5* ancestral haplotypes at 3p25 across clinically affected subjects identified key recombinations and reduced the disease region to a 2.36 Mb interval containing 20 annotated genes (Figures 3A and 3B; Table 1). Bidirectional resequencing of the 20 physical candidate *ARVD5* genes revealed 240 variants (Table S1 available online). Nineteen variants were found exclusively in clinically affected subjects on the mutation-screening panel, and 11 were determined to reside on the *ARVD5* ancestral haplotype through segregation analysis in AR14 (Table 2 and Figure 4). Screening of population controls showed that five of these variants were rare (<1% of the alleles screened; Table 2). Only one of the five, *TMEM43* 1073C→T (S358L) (Figure 5D), was shared by all clinically affected subjects across the 15 families and was retained on key recombinant *ARVD5* haplotypes identified in clinically affected subjects from families AR10 (Figure 6) and AR2 (data not shown). This suggested that *TMEM43* is *ARVD5*. Additional supportive evidence included clinically unaffected adult subjects who shared distal sections of the

Table 1. The 20 Physical Candidate Genes for *ARVD5*

Genes	Accession Number	MIM Number	Strand	Genomic Position Start	Genomic Position End	Exons
IQSEC1	NM_014869	610166	−	13003536	12917079	13
NUP210	NM_024923	607703	−	13436809	13332737	40
HDAC11	NM_024827	607226	+	13496824	13521834	10
FBLN2	NM_001004019	135821	+	13565625	13654922	18
WNT7A	NM_004625	601570	−	13896619	13835083	4
TPRXL	AK092426	611167	+	13953902	14082480	3
CHCHD4	NM_144636	611077	−	14141323	14128584	4
TMEM43	NM_024334	na	+	14141546	14160180	12
XPC	NM_004628	278720	−	14195143	14161651	16
LSM3	NM_014463	607283	+	14195341	14214840	4
SLC6A6	NM_003043	186854	+	14419110	14503973	15
GRIP2	NM_001080423	na	−	14558592	14510177	25
C3orf19	NM_016474	na	+	14668278	14689167	11
C3orf20	NM_032137	na	+	14691658	14789544	17
FGD5	NM_152536	na	+	14835810	14950899	20
NR2C2	NM_003298	601426	+	14964240	15065782	15
MRPS25	NM_022497	na	−	15081820	15065024	4
ZFYVE20	NM_022340	609511	−	15115659	15086584	14
CAPN7	NM_014296	606400	+	15222737	15269426	21
SH3BP5	NM_004844	605612	−	15349108	15271250	9
					Total	275

ARVD5 haplotype and lacked the *TMEM43* mutation (e.g., Figure 4). All available spouses (n = 47) and population controls (n = 161) were negative for the *TMEM43* mutation (416 mutation-negative chromosomes).

ARVC at Locus *ARVD5* Is Caused by a Missense Mutation in *TMEM43*

The longest isoform of *transmembrane protein 43* (*TMEM43*, GenBank accession number NM_024334) has 12 exons (Figure 5A) predicting a 400 amino acid protein that is 98% similar to the mouse protein (Figure 7). This conserved gene is found across all eukaryotic and prokaryotic species (Figure 7). We were able to extract full-length *TMEM43* cDNA from white blood cells and cardiac tissue from both patients and controls, demonstrating that *TMEM43* is expressed in both blood and cardiac tissue and that the *TMEM43* 1073C→T (S358L) mutation does not appear to affect splicing (Figures 5B, 5C, and 5E).

Bioinformatic analysis of TMEM43 predicts it to be a cytoplasmic membrane protein with several potential posttranslation modification sites (Figure 8). Unlike the transmembrane proteins of the desmosome (desmocollin and desmoglein), TMEM43 does not have a cadherin domain. Furthermore, protein sequence alignments with desmocollin and desmoglein show less than 10% identity and less than 12% similarity.[26] The mutation, S358L, occurs within the third predicted transmembrane domain and is highly conserved in mammalian, avian, amphibian, and insect orthologs (Figures 7A and 7B and Figure 8). Interestingly, a leucine at this position is found in the bacterium *Rhizobium loti*, but it is not found in any multicellular organisms (Figures 7A and 7B). The S358L mutation is predicted to be deleterious (Table 3), but little is known about the function of TMEM43 protein.

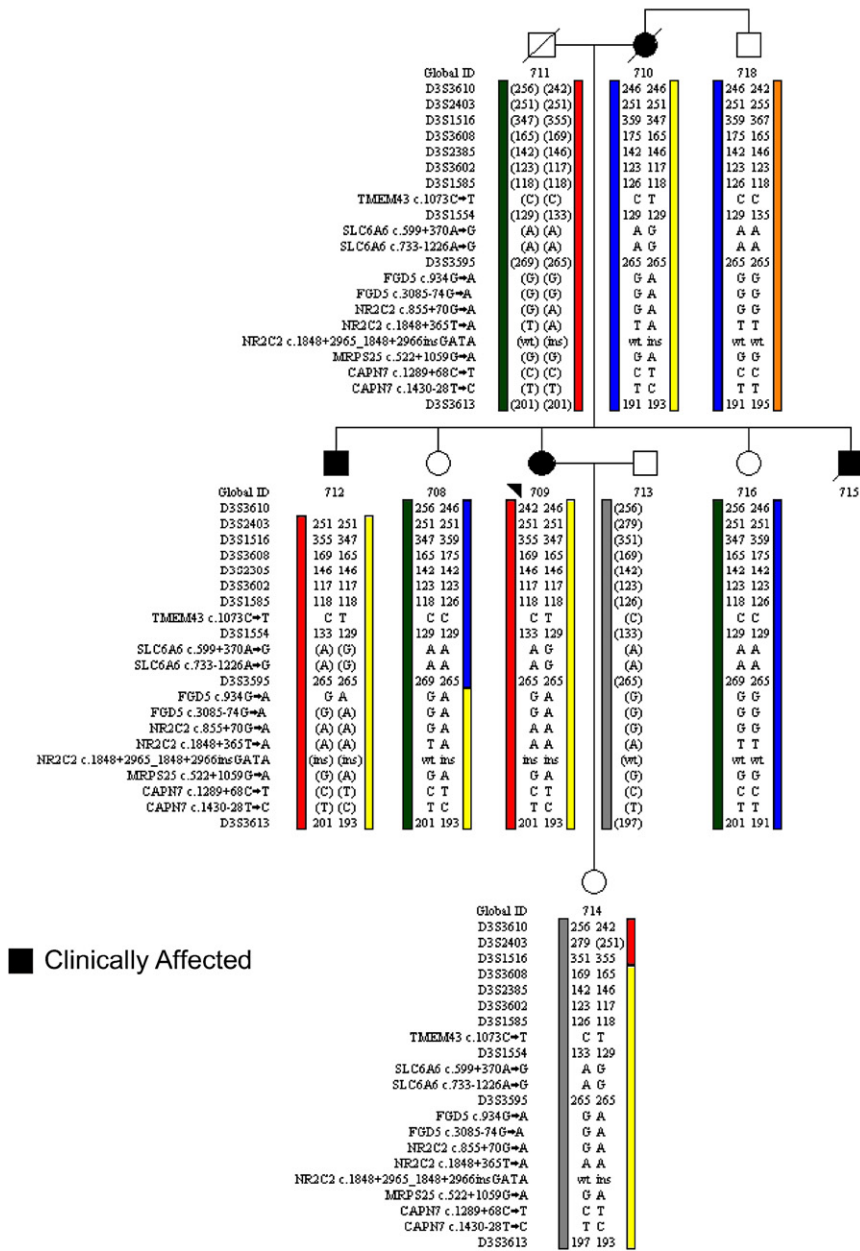### *TMEM43* Mutation Screening in *ARVD5* Linked Families

We sequenced genomic DNA from all available subjects born at a priori 50% risk (n = 295) across the 15 ARVC families for the presence of the 1073C→T *TMEM43* mutation. All clinically affected subjects (n = 83/83;

Table 2. Sequencing Variants Identified Exclusively in Clinically Affected Subjects

Gene Name	Accession number	Variant Nomenclature	Classification	Allele Frequency(# of chromosomes)	
HDAC11	NM_024827	c.369+18_369+19insG	Noncoding	nd	nd
TMEM43	*NM_024334*	*c.1073C→T*	*Missense (S > L)*	*0/322*	*0.00%**
TMEM43	NM_024334	c.1203+115T→C	Noncoding	nd	nd
XPC	NM_004628	c.2823+684G→C	Noncoding	nd	nd
SLC6A6	NM_003043	c.1-27420G→A	Noncoding	nd	nd
SLC6A6	NM_003043	*c.599+370A→G*	*Noncoding*	*dbSNP*	*46.00%*
SLC6A6	NM_003043	*c.733-1226A→G*	*Noncoding*	*50/90*	*55.60%*
FGD5	*NM_152536*	*c.934G→A*	*Missense (V > M)*	*2/318*	*0.60%**
FGD5	NM_152536	c.2186+22G→A	Noncoding	nd	nd
FGD5	NM_152536	c.2187-82G→A	Noncoding	nd	nd
FGD5	NM_152536	c.2220G→T	Synonymous (L > L)	nd	nd
FGD5	NM_152536	c.2613+50C→T	Noncoding	nd	nd
FGD5	*NM_152536*	*c.3085-74G→A*	*Noncoding*	*15/166*	*9.00%*
NR2C2	NM_003298	*c.855+70G→A*	*Noncoding*	*8/88*	*9.10%*
NR2C2	NM_003298	*c.1848+365T→A*	*Noncoding*	*16/90*	*17.80%*
NR2C2	NM_003298	*c.1848+2965_1848+2966insGATA*	*Noncoding*	*23/126*	*18.30%*
MRPS25	NM_022497	*c.522+1059G→A*	*Noncoding*	*0/138*	*0.00%**
CAPN7	NM_014296	*c.1289+68C→T*	*Noncoding*	*1/162*	*0.01%**
CAPN7	NM_014296	*c.1430-28T→C*	*Noncoding*	*0/160*	*0.00%**

Eleven of the 19 variants were determined to be in phase on the *ARVD5* ancestral haplotype and were subsequently sequenced on population controls (italics). The allele frequency of variants previously reported in the NCBI dbSNP was used. Rare variants (<1% of the alleles screened) are marked with an asterisk.

Subpedigree of AR14

Global ID	711	710	718
D3S3610	(256) (242)	246 246	246 242
D3S2403	(251) (251)	251 251	251 255
D3S1516	(347) (355)	359 347	359 367
D3S3608	(165) (169)	175 165	175 165
D3S2385	(142) (146)	142 146	142 146
D3S3602	(123) (117)	123 117	123 117
D3S1585	(118) (118)	126 118	126 118
TMEM43 c.1073C→T	(C) (C)	C T	C C
D3S1554	(129) (133)	129 129	129 135
SLC6A6 c.599+370A→G	(A) (A)	A G	A A
SLC6A6 c.733-1226A→G	(A) (A)	A G	A A
D3S3595	(269) (265)	265 265	265 265
FGD5 c.934G→A	(G) (G)	G A	G G
FGD5 c.3085-74G→A	(G) (G)	G A	G G
NR2C2 c.855+70G→A	(G) (A)	G A	G G
NR2C2 c.1848+365T→A	(T) (A)	T A	T T
NR2C2 c.1848+2965_1848+2966insGATA	(wt) (ins)	wt ins	wt wt
MRPS25 c.522+1059G→A	(G) (G)	G A	G G
CAPN7 c.1289+68C→T	(C) (C)	C T	C C
CAPN7 c.1430-28T→C	(T) (T)	T C	T T
D3S3613	(201) (201)	191 193	191 195

Global ID	712	708	709	713	716	715
D3S3610	256 246	256 246	242 246	(256)	256 246	
D3S2403	251 251	251 251	251 251	(279)	251 251	
D3S1516	355 347	347 359	355 347	(351)	347 359	
D3S3608	169 165	165 175	169 165	(169)	165 175	
D3S2305	146 146	142 142	146 146	(142)	142 142	
D3S3602	117 117	123 123	117 117	(123)	123 123	
D3S1585	118 118	118 126	118 118	(126)	118 126	
TMEM43 c.1073C→T	C T	C C	C T	(C)	C C	
D3S1554	133 129	129 129	133 129	(133)	129 129	
SLC6A6 c.599+370A→G	(A) (G)	A A	A G	(A)	A A	
SLC6A6 c.733-1226A→G	(A) (G)	A A	A G	(A)	A A	
D3S3595	265 265	269 265	265 265	(265)	269 265	
FGD5 c.934G→A	G A	G A	G A	(G)	G G	
FGD5 c.3085-74G→A	(G) (A)	G A	G A	(G)	G G	
NR2C2 c.855+70G→A	(A) (G)	G A	A A	(G)	T T	
NR2C2 c.1848+365T→A	(A) (A)	T A	A A	(A)	wt wt	
NR2C2 c.1848+2965_1848+2966insGATA	(ins) (ins)	wt ins	ins ins	(wt)	G G	
MRPS25 c.522+1059G→A	(G) (A)	G A	G A	(G)	C C	
CAPN7 c.1289+68C→T	(C) (T)	C T	C T	(C)	T T	
CAPN7 c.1430-28T→C	(T) (C)	T C	T C	(T)	201 191	
D3S3613	201 193	201 193	201 193	(197)		

■ Clinically Affected

Global ID	714
D3S3610	256 242
D3S2403	279 (251)
D3S1516	351 355
D3S3608	169 165
D3S2385	142 146
D3S3602	123 117
D3S1585	126 118
TMEM43 c.1073C→T	C T
D3S1554	133 129
SLC6A6 c.599+370A→G	A G
SLC6A6 c.733-1226A→G	A G
D3S3595	265 265
FGD5 c.934G→A	G A
FGD5 c.3085-74G→A	G A
NR2C2 c.855+70G→A	G A
NR2C2 c.1848+365T→A	A A
NR2C2 c.1848+2965_1848+2966insGATA	wt ins
MRPS25 c.522+1059G→A	G A
CAPN7 c.1289+68C→T	C C
CAPN7 c.1430-28T→C	T C
D3S3613	197 193

Figure 4. Segregation Analysis in Subpedigree AR14
Of the 19 variants found exclusively in clinically affected subjects on the mutation-screening panel, only 11 were found to reside on the *ARVD5* ancestral haplotype (yellow) through segregation analysis of clinically affected subjects (Global IDs 709, 710, and 712). Note that a clinically unaffected subject (Global ID 708) inherited a recombinant *ARVD5* haplotype from her clinically affected mother that lacks *TMEM43*. Alleles in brackets have been inferred.

Clinical Assessment of Penetrance in *ARVD5*

12 Lead ECG

Data on a total of 297 subjects (167 affected, 130 unaffected) that had at least one 12 lead surface ECG was available. Extended QRS >110 ms was significantly different between affected versus unaffected males (Table 4). Epsilon waves and T wave inversion commonly seen in ARVC[10] was seen in less than 3% of affected subjects in this cohort.

Holter Monitor

Of the 239 subjects (146 affected, 93 unaffected) with at least one Holter monitor report, the most prevalent feature was PVCs ≥200 and the presence of at least one run of nonsustained VT (Table 4), both significantly more common in affected than unaffected subjects.

Echocardiography

Of the 244 subjects (143 affected, 101 unaffected) that had at least one 2D echocardiogram available for analysis, the most prevalent feature was LVE based on LVEDD (Table 4),[32,36] and LVE was significantly different between affected and unaffected subjects.
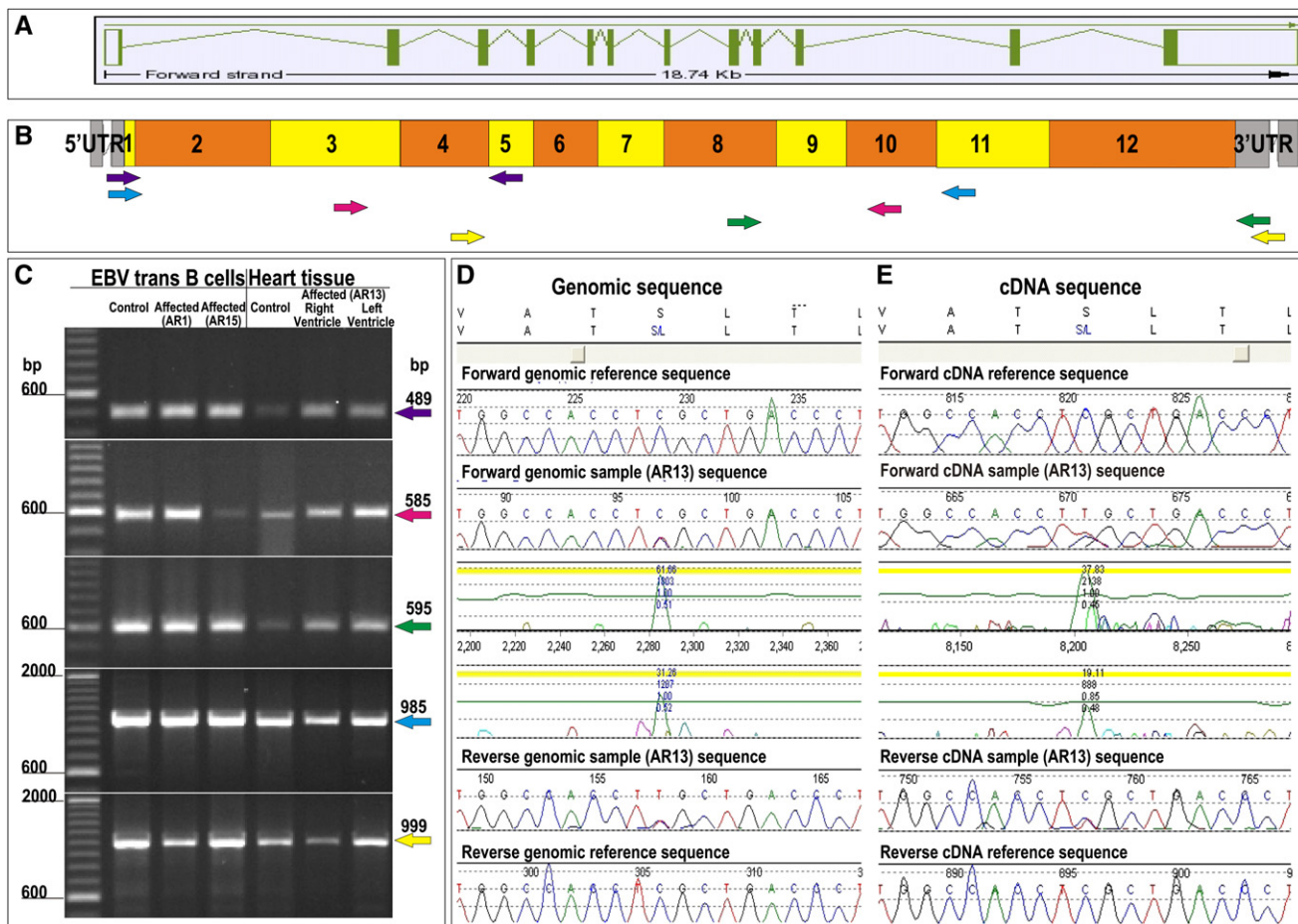
SAECG

Of the 198 subjects (97 affected, 101 unaffected) who had at least one SAECG available for analysis, all late potentials were significantly more common in affected versus unaffected males; only QRS was significant in females (Table 4).

ARVC Penetrance

We used 137 subjects (60 affected males, 77 affected females) to determine penetrance. Median age to develop an *ARVD5* associated phenotype was 32 years (95% CI 28–35) for males and 44 years (95% CI 39-48) for females,

40 males, 43 females) and 28.8% of clinically unaffected subjects (n = 61/212; 18 males, 43 females) were mutation carriers (Figure 2). Interestingly, 57% (35/61) of unaffected mutation carriers were found on subsequent testing to have clinical signs of ARVC. These included ectopy on Holter monitor (≥200 PVCs over 24 hr[11]), extended QRS on 12 lead ECG or SAECG,[10] or an enlarged left ventricle (>2 SD above the mean). The remaining 26 clinically unaffected mutation carriers, 3 males and 23 females, were at a median age of 22 years and 33 years respectively. The 151 subjects who had no clinical signs and who did not have the *TMEM43* variant were considered unaffected (Figure 2).

Figure 5. Gene Structure and Mutation Analysis of *TMEM43*

(A) Gene structure of *TMEM43*. Exons are represented by boxes. Translated exons are solid green, and untranslated exons are clear. Introns are represented by green lines.

(B) Coverage of primers designed to amplify cDNA showing position of PCR primer pairs: exons 1–4 (purple), exons 4–9 (red), exons 9–12 (green), exons 1–10 (blue), and 5–12 (yellow).

(C) PCR products amplified from cDNA of EBV-transfected B cells of affected subjects from the mutation-screening panel (affected AR1 and affected AR15) and unaffected (control) subjects. cDNA of heart tissue from both the left and right ventricle of an affected subject (AR13) and a heart biopsy from a control subject were size fractionated by electrophoresis.

(D and E) Forward and reverse sequencing traces showing the *TMEM43* 1073C→T mutation of an affected subject's (AR13) genomic and cDNA. The amino acid translations (top) shows the S358L amino acid substitution.

with 100% of males and females penetrant by 63 and 76 years, respectively (Figure 9 and Table 5). Males were twice as likely to reveal the disease phenotype than females (RR 2, 99% CI 1.2–3.3) (p ≤ 0.0001). The commonest clinical features for which subjects were initially penetrant were ectopy (44%) and LVE (27%), and those were followed by VT (9%), QRS >110 ms (9%), late potentials on SAECG (7%), SCD (2%), and heart failure (2%).

Heart Failure

Fourteen of 89 affected males developed heart failure at a median age of 63 years (95% CI 41–84 years) compared with no unaffected males (p ≤ 0.0001, log rank) (Figure 10A$_i$). One unaffected female at age 79 developed heart failure compared to seven affected females (median age 73 years, 95% CI 69–77 years) (p ≤ 0.001, log rank)

(Figure 10A$_{ii}$). Affected males were three times more likely to develop heart failure than females (RR 3.4, 95% CI 1.36–8.57, p ≤ 0.009) (Figure 10A$_{iii}$).

Life Expectancy

In affected subjects (n = 257), there were 123 (48%) deaths (99 males and 24 females). Sudden cardiac death occurred in 86% (86/99) of the males and 42% (10/24) of the females. Survival was significantly reduced in affected versus unaffected subjects. Median survival was 41 compared to 83 years (p ≤ 0.0001, log rank) in affected versus unaffected males (Figure 10B$_i$) and 71 compared to 83 years in affected versus unaffected females (p ≤ 0.002, log rank) (Figure 10B$_{ii}$). The relative risk of dying was 6.8 times greater in affected males versus affected females (95% C.I: 4.3–10.9, p ≤ 0.0001) (Figure 10B$_{iii}$).

Subpedigree of AR10

Global ID — 795

	795
D3S3610	(246)
D3S2403	(251)
D3S1516	(331)
D3S3608	(165)
D3S2385	(142)
D3S3602	(123)
D3S1585	(126)
TMEM43 c.1073C→T	(C)
D3S1554	(129)
D3S3595	(265)
FGD5 c.934G→A	(G)
MRPS25 c.522+1059G→A	(G)
CAPN7 c.1289+68C→T	(C)
CAPN7 c.1430-28T→C	(T)
D3S3613	(201)

Global ID — 794

	794	
D3S3610	246	256
D3S2403	251	259
D3S1516	347	331
D3S3608	165	175
D3S2385	146	146
D3S3602	117	123
D3S1585	118	118
TMEM43 c.1073C→T	T	C
D3S1554	129	129
D3S3595	265	265
FGD5 c.934G→A	G	G
MRPS25 c.522+1059G→A	G	G
CAPN7 c.1289+68C→T	C	C
CAPN7 c.1430-28T→C	(T)	(T)
D3S3613	195	195

799 * 800

Global ID — 796

	796	
D3S3610	246	246
D3S2403	251	251
D3S1516	331	347
D3S3608	165	165
D3S2385	142	146
D3S3602	123	117
D3S1585	126	118
TMEM43 c.1073C→T	C	T
D3S1554	129	129
D3S3595	265	265
FGD5 c.934G→A	G	G
MRPS25 c.522+1059G→A	G	G
CAPN7 c.1289+68C→T	C	C
CAPN7 c.1430-28T→C	T	T
D3S3613	201	195

■ Clinically Affected

Figure 6. Segregation Analysis in Subpedigree AR10
Clinically affected subjects (Global IDs 794 and 796) only have one of the five rare variants because of a historical recombination event on the *ARVD5* haplotype (yellow). Alleles in brackets have been inferred.

601487).[37] A genome-wide scan for peroxisome proliferator response elements (PPREs) identified 1085 potential target genes of PPARγ, including *TMEM43*.[38] If TMEM43 is a part of an adipogenic pathway regulated by PPARγ, then perhaps dysregulation of this pathway may explain the fibrofatty replacement of the myocardium in ARVC patients.

Studies that assess the ARVC phenotype in patients with mutations in *plakophilin-2*,[39] *desmoglein-2*,[16] and *plakoglobin*[2] typically evaluate clinical features in mutation carriers only. The assumption is that all cardiac signs present in affected subjects are due to the underlying genetic defect. However, it may be that some signs are due to the genetic background of either the family or the source population. The size of the ARVC cohort in this study provides an opportunity to compare and define ARVC-specific clinical features in affected and unaffected subjects.

By using the benefit of a control group, we evaluated modified clinical criteria[11] in affected versus unaffected subjects. We included LVE, a clinical feature that is noted in other ARVC studies[40–44] and that is prevalent in this cohort. For all clinical features examined, a significant difference was found between affected versus unaffected males. Females were significantly different for all features with the exception of RMS and LAS on SAECG and a QRS >110 ms on 12 lead ECG.

For the penetrance study, we followed subjects who were alive at the start of the study (1996), who had a medical record, and who had a genetic diagnosis (mutation carriers or obligate carriers). The results showed that ARVC was fully penetrant in males by the age of 63 and in females by the age of 76; ectopy and LVE were often the first presenting features, and few presented with death or heart failure. Therefore, in order to assess the penetrance of heart failure and death, we used all affected subjects. Heart failure and death were morbid outcomes at early ages in both males and females, with far more serious early events in males. In both sexes, heart failure occurs as a later
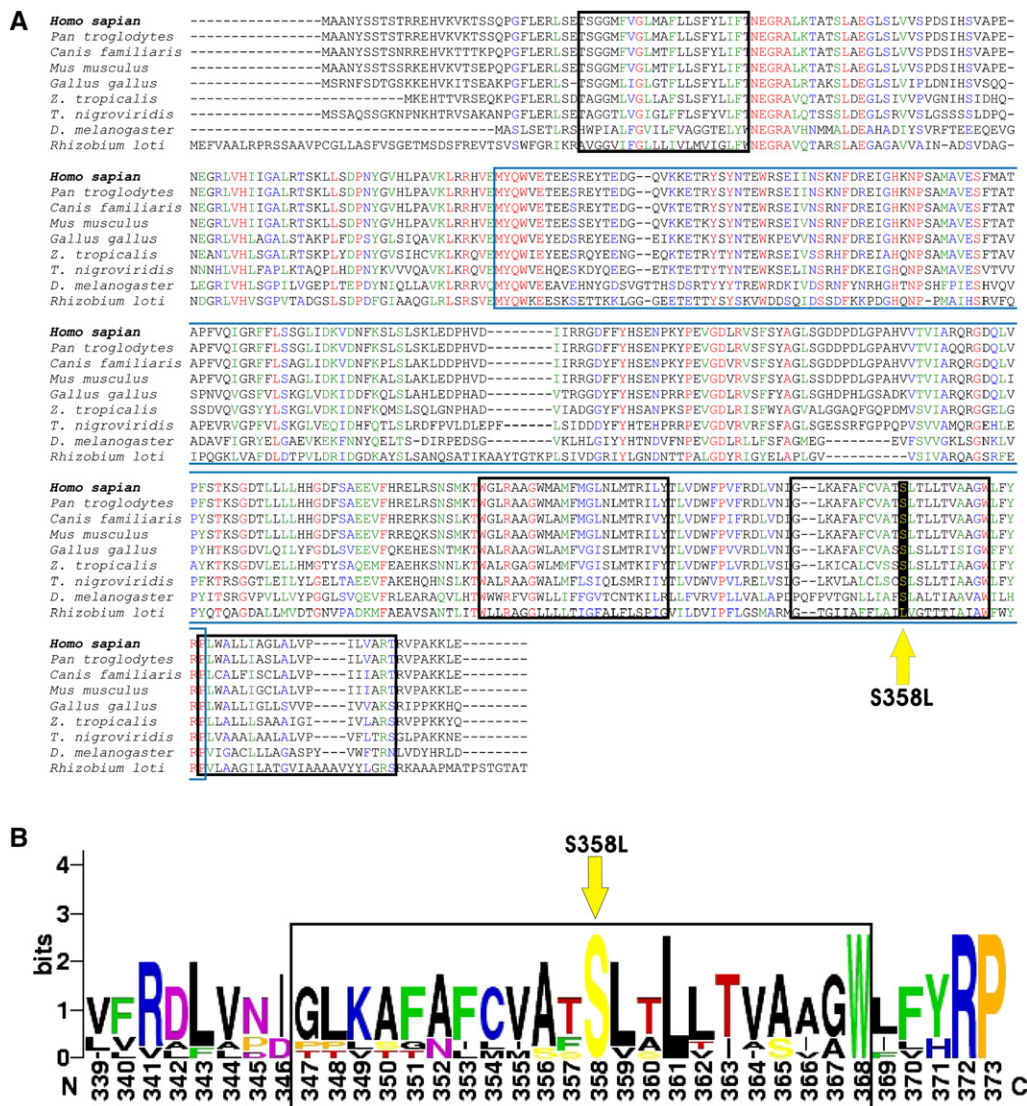
Discussion

Mutations in desmosomal genes underlie several genetic subtypes of ARVC, suggesting that ARVC is primarily a disease of the desmosome.[5,7] We used a positional mapping approach and 15 ARVC families that shared a disease-associated haplotype on 3p from a genetic isolate to identify *ARVD5*. All clinically affected subjects that were screened for the *TMEM43* 1073C→T mutation (n = 83) were mutation carriers. This mutation was not detected in spouses or population controls. The *TMEM43* gene is expressed in cardiac tissue and is evolutionarily conserved, and the amino acid substitution is predicted to be deleterious.

Recently, signaling pathways have been implicated in ARVC pathogenesis.[7,37] For example, plakoglobin, when freed from desmosomal complexes, translocates to the nucleus where it competes and opposes the action of β-catenin and downregulates the canonical Wnt/β-catenin signaling pathway.[37] Suppression of the canonical Wnt/β-catenin signaling upregulates two adipogenic transcription factors, C/EBP-α (MIM 116897) and PPARγ (MIM

Figure 7. Multiple Alignment of the *TMEM43* Gene across Eight Eukaryotic and Prokaryotic Species
(A) Clustal W align was used to align orthologs from *Homo sapiens* (NP_077310), *Pan troglodytes* (XP_516299), *Canis familiaris* (XP_541751), *Mus musculus* (NP_083042), *Gallus gallus* (XP_414378), *Zenopus tropicalis* (UP10004D5297), *Tetraodon nigroviridis* (Q4RXL8), *Drosophila melanogaster* (NP_64162), and *Rhizobium loti* (Q98HF3).[25] The blue box outlines the DUF1625 domain, and the black boxes outline predicted transmembrane domains. Completely conserved residues are red, strongly similar residues are green, and weakly similar residues are blue. The S358L mutation is marked by a yellow arrow.
(B) The web logo format was used for aligning eukaryotic species. The third transmembrane domain is outlined (black box). The S358L mutation is marked by a yellow arrow.

manifestation in subjects who did not succumb to SCD. These major manifestations define ARVC, because of the *TMEM43* mutation, as a lethal, fully penetrant, sex-influenced, autosomal-dominant disorder.

Biases exist in studies that use both retrospective and prospective data; the use of well-ascertained sibships may mitigate some of these. Although formal assessment of right ventricular structure is not available for the majority of subjects in this cohort, the cardiomyopathy fulfills diagnostic criteria for ARVC,[11] it has been historically diagnosed as ARVC,[17,19] and fibrofatty replacement of the myocardium is present. The involvement of the left ventricle in this cohort supports the recent call to place ARVC within a group called "arrhythmic cardiomyopathies," which would take into account various presentations of disease.[45] These findings may not be applicable to other forms of inherited ARVC. However, it is likely that mutations in *TMEM43* are present in other populations. Issues of classification in the cardiomyopathies in general will be solved by their underlying genetic etiology; thus, disease-specific genotype/phenotype information will become the most useful and relevant.

This study has the advantage of having access to past medical records and extensive genealogical data, and a single cardiac-care team followed all subjects. Clinical signs due to the genetic background of either the family or the
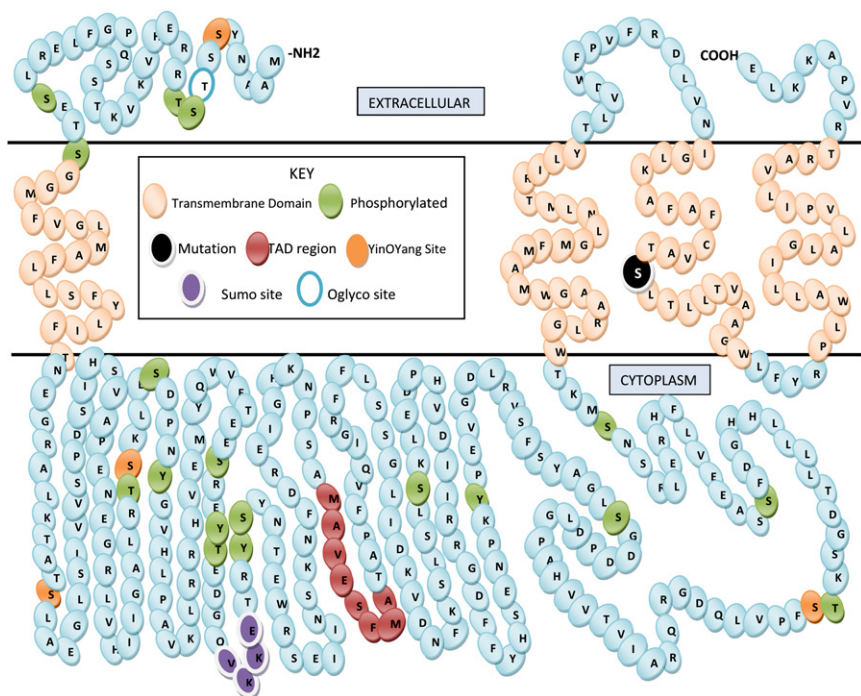
Figure 8. Predicted Topography of the TMEM43 Protein
Indicated are transmembrane domains (beige), phosphorylation sites (green), a transactivation domain (red), YingOYang sites (orange), a SUMO attachment site (purple), and an O-glycosylation site (blue open). The extracellular and cytoplasmic regions may be reversed: There is evidence supporting either orientation.

source population were controlled for by the use of unaffected subjects from well-ascertained sibships. This genetic subtype does not show the typical ARVC ECG findings of T wave inversion or epsilon waves.[10] A direct benefit of this process therefore has been the provision of accurate mutation-specific clinical data for precise genetic counseling and follow-up of those at-risk.[20]

We have identified the gene that causes ARVC at locus *ARVD5*. A missense mutation in *TMEM43* causes a fully penetrant, sex-influenced lethal form of ARVC. Although little is known about the function of this gene, it encodes a transmembrane protein that could be a target of PPARγ, and this may explain the fibrofatty replacement of the myocardium in ARVC patients. Future directions include functional studies of the TMEM43 protein.

Supplemental Data

One table is available at http://www.ajhg.org/.

Acknowledgments

Table 3. Prediction of the *TMEM43* 1073C→T, S358L, Mutation Effect

Program	SIFT	Panther	PolyPhen	SNPs3D	PMut
S358L mutation	deleterious	deleterious	Benign	deleterious	deleterious

Five different bioinformatic programs were used for predicting the effect of the S358L mutation. Note that sequence homology used by the PolyPhen analyses was calculated with alignments of orthologs from Eukaryota and bacteria.

Web Resources

The URLs for data presented herein are as follows:

NCBI dbSNP, http://www.ncbi.nlm.nih.gov/sites/entrez?db=snp&cmd=search&term=

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim

UCSC Genome Browser, http://genome.ucsc.edu/

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Table 4. Prevalent Cardiac Features on First Clinical Test

| 12 Lead ECG	Affected			Unaffected			Affected Males versus Unaffected Males, χ^2	Affected Females versus Unaffected Females, χ^2
	Male	Female	Total	Male	Female	Total		
Subjects Tested (N)	78	89	167	64	66	130		
Mean Age (SD)	30.0 (12.1)	37.7 (15.8)		33.1 (16.0)	38.2 (13.2)			
QRS >110 ms n (%)	25 (32)	8 (9)	33 (19.8)	4 (6)	2 (3)	6 (4.6)	$p \leq 0.001$	$p \leq 0.2$ ns
Holter Monitor								
Subjects Tested (N)	67	79	146	49	44	93		
Mean Age (SD)	31.1 (13.3)	37.7 (15.5)		33.1 (15.9)	38.2 (12.8)			
PVCs ≥200/24 hr n (%)	47 (70)	47 (59)	94 (64.4)	0 (0)	1 (2)	1 (1.1)	$p < 0.001$	$p < 0.001$
≥1 run ns VT n (%)	13 (19)	18 (23)	31 (21.2)	0 (0)	0 (0)	0 (0.0)	$p < 0.01$	$p < 0.001$
Echocardiograph								
Subjects Tested (N)	67	76	143	50	51	101		
Mean Age (SD)	31.6 (13.2)	39.6 (15.1)		32.5 (14.9)	38.3 (13.3)			
LVE >2 SD n (%)	35 (52)	27 (20)	62 (43.3)	10 (20)	4 (8)	14 (13.9)	$p < 0.001$	$p < 0.001$
SAECG								
Subjects Tested (N)	32	65	97	50	51	101		
Mean Age (SD)	30.9 (14.5)	39.8 (14.5)		36.7 (16.3)	38.9 (11.0)			
QRS >114 ms n (%)	19 (59)	19 (29)	38 (39.2)	10 (20)	2 (4)	12 (11.9)	$p \leq 0.001$	$p \leq 0.001$ ns
RMS <20 ms n (%)	15 (47)	18 (28)	33 (34.0)	12 (24)	9 (18)	21 (20.8)	$p \leq 0.05$	$p \leq 0.3$ ns
LAS >38 ms n (%)	17 (53)	21 (32)	38 (39.2)	14 (28)	13 (25)	27 (26.7)	$p \leq 0.025$	$p \leq 0.5$ ns

This table shows 12 lead ECG, Holter, echocardiographic, and SAECG manifestations used in penetrance analysis in subjects from 15 families with ARVC due to a mutation in *TMEM43*. Abbreviations are used as follows: PVC, premature ventricular complex; VT, ventricular tachycardia; LVE, left ventricular enlargement indexed to height and weight; RMS, root mean square voltage of the terminal 40 ms of filtered QRS; and LAS, low-amplitude signals.

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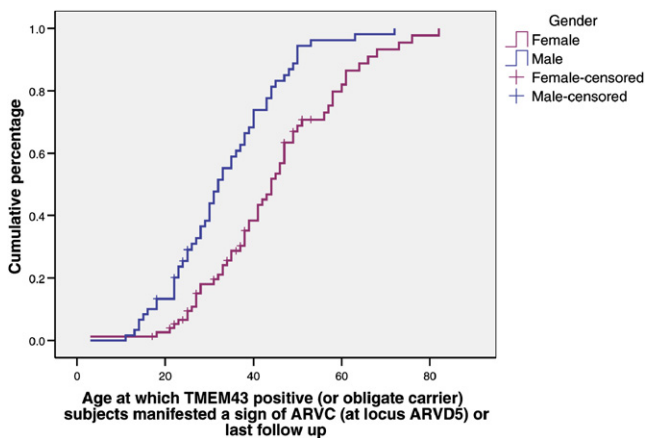
**Figure 9. ARVC Penetrance Caused by the *TMEM43* 1073C→T Mutation**

Cumulative proportion by age at which male (n = 60) and female (n = 77) mutation positive affected subjects manifested a first clinical sign of ARVC. Subjects were censored at last follow-up.

Table 5. Percentage of Subject's Penetrant by Each Age

Age (years)	11–20	21–30	31–40	41–50	51–60	61–70	71–80
Males	13	39	68	89	96	100	100
Females	3	18	38	67	80	97	100

This table shows the cumulative percentage of subjects penetrant by decade.
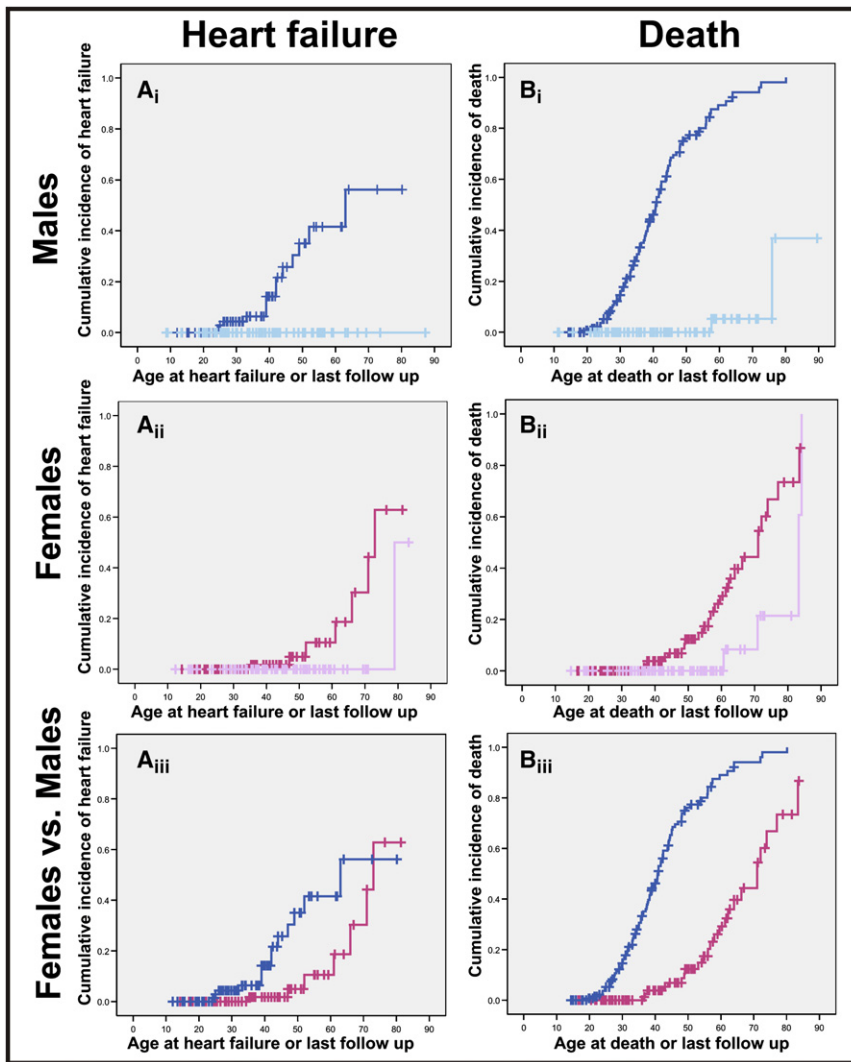
Figure 10. Time to Event Analysis of Heart Failure and Death in Affected Subjects

(A$_i$–A$_{iii}$) Cumulative incidence of heart failure; panels describe the following: (A$_i$) affected males (n = 89, dark blue) compared to unaffected males (n = 71, light blue) (p ≤ 0.0001: log rank); (A$_{ii}$) affected females (n = 87, dark pink) compared to unaffected females (n = 68, light pink) (p ≤ 0.001, log rank); and (A$_{iii}$) affected males (dark blue) versus affected females (dark pink).

(B$_i$–B$_{iii}$) Life expectancy; panels describe the following: (B$_i$) time to death in affected males (n = 148, dark blue) and unaffected males (n = 77, light blue); (B$_{ii}$) time to death in affected females (n = 109, dark pink) and unaffected (n = 74, light pink); and (B$_{iii}$) affected males (dark blue) versus affected females (dark pink).

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