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Cadherin 2-Related Arrhythmogenic Cardiomyopathy: Prevalence and Clinical Features

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Running title: Cadherin 2-Related Arrhythmogenic Cardiomyopathy

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ABSTRACT

Background. Arrhythmogenic cardiomyopathy (ACM) is an inherited cardiac disease characterized by fibro-fatty replacement of the right and/or left ventricle, often causing ventricular dysfunction and life-threatening arrhythmias. Variants in desmosomal genes account for up to 60% of cases. Our objective was to establish the prevalence and clinical features of ACM stemming from pathogenic variants, in the non-desmosomal cadherin 2 (*CDH2*), a novel genetic substrate of ACM.

Methods. A cohort of 500 unrelated patients with a definite diagnosis of ACM and no disease-causing variants in the main ACM genes was assembled. Genetic screening of *CDH2* was performed through next-generation or Sanger sequencing. Whenever possible, cascade screening was initiated in the families of *CDH2*-positive probands, and clinical evaluation was assessed.

Results. Genetic screening of *CDH2* led to the identification of 7 rare variants: five, identified in 6 probands, were classified as pathogenic or likely pathogenic. The previously established p.D407N pathogenic variant was detected in 2 additional probands. Probands and family members with pathogenic/likely pathogenic variants in *CDH2* were clinically evaluated, and along with previously published cases, altogether contributed to the identification of gene-specific features (13 cases from this cohort and 11 previously published, for a total of 9 probands and 15 family members). Ventricular arrhythmic events occurred in most *CDH2*-positive subjects (20/24, 83%), while the occurrence of heart failure was rare (2/24, 8.3%). Among probands, sustained ventricular tachycardia and/or sudden cardiac death occurred in 5/9 (56%).

Conclusions. In this worldwide cohort of previously genotype-negative ACM patients, the prevalence of probands with *CDH2* pathogenic/likely pathogenic variants was 1.2% (6/500).

Our data show that this cohort of *CDH2*-ACM patients has a high incidence of ventricular arrhythmias, while evolution toward heart failure is rare.

Key words: Arrhythmogenic cardiomyopathy, cadherin 2, ventricular tachycardia, sudden cardiac death, genetic variant, next generation sequencing

Non-standard Abbreviations and Acronyms

ARVC: arrhyhtmogenic right ventricular cardiomyopathy

SCD: sudden cardiac death

RV: right ventricle

LV: left ventricle

ACM: arrhythmogenic cardiomyopathy

MAF: minor allele frequency

VUS: variant of unknown significance

PVC: premature ventricular complexes

EC: ectodomain

VT: ventricular tachycardia

NSVT: non sustained ventricular tachycardia

ICD: internal cardioverter defibrillator

DCM: dilated cardiomyopathy

ACMG: American College of Medical Genetics and Genomics

CI: confidence interval

LoF: loss of function

EP: electrophysiological study

CKO: conditional knockout

ACOG: agenesis of corpus callosum, axon pathfinding, cardiac, ocular, and genital defects

FM: family member

WGS: whole-genome sequencing

WES: whole-exome sequencing

NGS: next generation sequencing

SNP: single nucleotide polymorphism

MI: myocardial infarction

HF: heart failure

CVA: cerebrovascular accident

TW: T wave

TWI: T wave inversion

LVEF: left ventricular ejection fraction

RVEF: right ventricular ejection fraction

LBBB: left bundle-branch block

BCE: breakthrough cardiac event

INTRODUCTION

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is an inherited cardiac disease characterized by fibro-fatty replacement of the right ventricle, accompanied by electrical instability predisposing to ventricular arrhythmias and sudden cardiac death (SCD).[1,2] The major form of the disease (ARVC) is characterized by a predominant right ventricular (RV) involvement. However, given the evidence of forms with bi-ventricular or predominant left ventricular (LV) involvement,[3] the term arrhythmogenic cardiomyopathy (ACM) is now preferred.[4,5]

ACM is mainly caused by variants in genes encoding proteins of the desmosome (*PKP2, DSP, DSG2, DSC2, JUP*), a specialized junction involved in cell adhesion.[5,6] Desmosomes, together with the fascia adherens junctions and the gap junctions, are located at the intercalated discs, which maintain a tight connection between cardiomyocytes and allow cellular communication. In mammals, these 3 functional structures are found in close proximity,[7] and form a hybrid zone that operates as a single functional network, known as the area composita.[8,9] Nowadays, the idea of ACM being a disease whose functional basis extends beyond the desmosome to the wider zone of the intercalated discs, is gaining interest.[10,11] In a previous study by Mayosi et al, we identified clinically actionable variants in the *CDH2* gene, encoding cadherin 2, a major structural component of fascia adherens junctions, as a novel genetic substrate of ACM.[12,13] The concept that the genetic basis of ACM extends to non-desmosomal protein components of the intercalated disc, has been further demonstrated by two other studies linking the ACM phenotype to variants in the *CTNNA3*,[14] and *TJP1* genes.[15]

These initial discovery studies[12,14,15] highlighted the potential contribution of components of the intercalated disc to the pathogenesis of ACM, thus widening its genetic basis. However, these findings require validation in large ACM patient cohorts, to allow correct estimates of

their relative contribution, prevalence, and clinical characteristics. To this end, we have collected the largest available worldwide population of previously genotype-negative ACM probands (n=500) to validate the contribution of *CDH2* variants to ACM and to establish a reliable prevalence. As a second step, we performed, whenever possible, cascade screening in the families of *CDH2* positive probands. Finally, to identify gene-specific features, we analyzed all *CDH2*-positive probands and affected family members from this and previously published studies,[12,13] for a total of 9 probands and 15 family members.

METHODS

All data, analytical methods, and study materials supporting this study are available from the corresponding author on reasonable request for purposes of reproducing the results or replicating the procedures. The study was approved by the local Ethics institutional Review Boards, and the participants gave informed consent. A full description of the methods is provided in the Data Supplement.

RESULTS

Study population

Most of the 500 patients were Caucasian (n=416; 83%), followed by Han Chinese (n=77; 15%) and African-American (n=2). In 5 cases, ethnicity was unknown. Males were 59% and females 41%.

Genetic findings in CDH2

We identified 7 rare genetic variants (MAF<0.0001) in *CDH2*, 6 missense and 1 frameshift, in 8 unrelated subjects (Tables 1-2; Figure 1-Panel A). Among these, one variant was classified as pathogenic (p.D407N), 4 as likely pathogenic (p.P205L, p.N261D, p.N658Kfs*3, p.P679A), and 2 as variants of unknown significance (VUS) (Table 1). Cascade family

screening was feasible in 4 *CDH2*-proband's families (p.P205L, p.N261D, p.D407N and p.P679A), for a total of 12 subjects (7 variant-positive and 5 variant-negative). An additional family member, affected by ACM, died suddenly before genetic testing could be performed.

The p.D407N pathogenic variant, previously identified in 5 affected subjects of 2 independent studies,[12,13] was also detected in 2/500 unrelated subjects comprising this study cohort. Both probands satisfied major ACM diagnostic criteria, such as structural dysfunction (important dilatation of RV) and depolarization/repolarization abnormalities (Table 2). One of the two p.D407N probands, symptomatic for arrhythmic events, had three affected relatives, one brother who died suddenly at age 18 years, another brother and a nephew (both carrying the p.D407N variant) with RV involvement and frequent premature ventricular complexes (PVCs) despite beta-blocker therapy.

The p.N658Kfs*3 frameshift variant, resulting from a nucleotide duplication that putatively results in premature termination of the protein, is the first non-missense variant thus far described in *CDH2*–associated ACM. The patient with this likely pathogenic variant is a female, diagnosed at 59 years, with dilatation of the RV, accompanied by depolarization/repolarization abnormalities and frequent PVCs (>500 per 24 hours, Table 2).

The other three variants classified as likely pathogenic (p.P205L, p.N261D and p.P679A) are ultra-rare missense variants (0 or 1 allele in gnomAD), located in the extracellular region of the protein (Figure 1-Panel A), with evidence in support of pathogenicity coming also from genotype-phenotype correlation data in the respective patient families (Figure 2). The p.N261D variant affects the Ca^{2+}-binding site in the ectodomain 1-2 (EC1-EC2)-inter-domain that plays a key role in cadherin-cadherin adhesion.[20] This variant was identified in a proband with sustained ventricular tachycardia (VT), implanted with an ICD at age 18 years, in one affected brother with left bundle branch block, non-sustained VTs and very frequent PVCs (40,000/24 h), and in a cousin with signs of non-compaction and an episode of heart failure

triggered by supraventricular tachycardia (Figure 2). The p.P679A positive individual is a female who presented with syncope at age 20 years and who eventually developed biventricular structural impairment (Table 2). The variant was also present in her affected father, while it was absent in her unaffected sibling (Figure 2). The p.P205L variant resides in the EC1 domain that, together with EC2, is considered an essential structural element for cadherin-mediated intercellular adhesion.[21] This variant was identified in a male proband who presented with repolarization abnormalities and non-sustained VTs. Cascade family screening also identified the variant in the affected father, while it was absent in 3 unaffected family members. A brother, positive for the same variant, was only partially investigated, and at present he cannot be clinically classified (Table 2; Figure 2).

Finally, two rare variants were classified as VUSs due to lack of a high degree of conservation of the affected residue, few clinical information available and the presence of the variant in exome/genome datasets, albeit with MAFs much lower than the disease prevalence (Table 1).[22]

Pathogenic/likely pathogenic variants in other genes associated with arrhythmogenic and/or dilated cardiomyopathy (DCM) were identified in only 3 probands not carrying any *CDH2* variant (Supplemental Table 1).

In conclusion, 7 rare variants in *CDH2* were identified in 8 out of 500 ACM unrelated patients (1.6%; 95% CI 0.5-2.7%) who lack a pathogenic/likely pathogenic variant in the major ACM genes. Considering only the 6 patients with ACMG-graded pathogenic/likely pathogenic variants, the prevalence of *CDH2* clinically actionable variants in this ACM cohort is 1.2% (6/500) (95% CI 0.2-2.2%).

***CDH2* genetic variability and variant location**

Since *CDH2* is a novel human disease gene and in order to aid the eventual classification of genetic variants, it could be useful to evaluate its natural genetic variability. To this end, we took advantage of the publicly-available exome/genome datasets including more than 140,000 subjects.

CDH2 is a gene extremely intolerant to loss-of-function variation (LoF), as indicated by the constraint metrics in the gnomAD database, with a high discrepancy between the number of expected (n=41) and observed (n= 6) LoF variants.

At the same time, the *CDH2* gene, as many other genes, seems to be more tolerant to missense variation, with 74% of the theoretically expected variants being actually observed in the gnomAD population.[16] However, the number of subjects with rare (MAF<0.0001) and potentially clinically-relevant missense variants differs significantly between the ACM cohort (pathogenic/likely pathogenic, 5/500, 1.0%) and the gnomAD population (potentially pathogenic, 300/140,000, 0.2%) (p=0.0010). This difference becomes even more striking when considering only the ultra-rare (MAF<0.000005) and potentially clinically-relevant missense variants, (pathogenic/likely pathogenic, 5/500, 1.0% in ACM cases vs potentially pathogenic, 59/140,000, 0.04% in gnomAD) (p<0.0001).

Regarding the localization within the protein of the pathogenic/likely pathogenic amino acid substitutions identified in our patient cohort, they exclusively reside in the extracellular region of cadherin 2 that appears intolerant to missense variation according to the computational analysis by MetaDome (Figure 1-Panel B).[19] Specifically, most of these variants cluster in the head EC1-EC3 domains.

CDH2-associated ACM clinical features

To identify gene-specific clinical features, we considered all probands with *CDH2* pathogenic and likely pathogenic variants of the present (n=6) and of the two previously published studies (n=3)[12,13] along with their 15 affected family members with complete clinical information (7 from this and 8 from previously published studies), for a total of 24 patients with *CDH2*-associated ACM (Table 3).

Sustained VTs and SCD occurred in 5/9 probands (56%). All except one of the remaining index cases had frequent PVCs and/or non-sustained VTs, with an ICD implanted in 6 (67%) (Table 3). The only proband with no spontaneous arrhythmias had VT induction at the EP study and recurrent episodes of heart failure. None of the other probands had heart failure episodes and none underwent a heart transplant during a mean follow-up period of 13.5±8.5 years. Regarding the structural alterations observed, all probands had right ventricular involvement, with a biventricular involvement observed in 3 (33%).

Among the 15 clinically affected *CDH2*-postive family members, 2 suffered SCD and 10 had frequent PVCs and/or non-sustained VTs (Table 3). Left ventricular involvement was present in 4 genotype-positive family members and in only one of them heart failure was reported during supraventricular tachycardia.

Discussion

The present report provides the first assessment of the prevalence of *CDH2* clinically actionable variants in ACM, and establishes its potential clinical features. *CDH2* encodes cadherin 2, also known as N-cadherin, the main protein component of fascia adherens junctions at the intercalated disc. The main novel finding of this study is that pathogenic/likely pathogenic variants in *CDH2* are responsible for 1.2% of genetically elusive ACM cases (95% CI 0.2-2.2%). Secondly, the CDH2-p.D407N, found in 9 ACM patients, appears to be the most common variant associated with *CDH2*-mediated ACM.

In addition, this study shows the presence of two rather specific clinical features for this cohort of *CDH2*-ACM. One is that left ventricular involvement was present in one third of patients, with a rare evolution toward heart failure despite adequate follow-up period. The second is that the majority of *CDH2*-positive ACM patients have a ventricular arrhythmic phenotype (20/24, 83%), an observation with both prognostic and therapeutic implications.

Prevalence of CDH2 variants in ACM and clinical implications

After the initial discovery of *CDH2* variants as a novel genetic substrate of ACM,[12] a finding soon after replicated in another study,[13] the need of estimating the contribution of *CDH2* variants to ACM pathogenesis in a large patient cohort emerged. Therefore, we have collected a cohort of 500 genetically-negative ACM patients and performed genetic analysis of the *CDH2* gene. The prevalence of *CDH2* clinically actionable variants identified in this cohort is 1.2% (95% CI 0.2-2.2%), similar to our original estimation in the discovery study.[12] The yield of genetic testing in ACM is estimated to be close to 60%,[23,24] and therefore, a significant number of patients remains genetically-elusive. Although the prevalence of *CDH2* variants in ACM is low, this finding is not without potentially relevant clinical implications.

Indeed, the thorough clinical evaluation of those patients with *CDH2*-ACM enabled the identification of few gene-specific features. Specifically, some of the pathogenic variants so far identified, such as the p.Q229P[12] and the original p.D407N,[12,13,this study] seem to be associated with a severe arrhythmic phenotype, with recurrent sustained ventricular tachycardia and SCD. It seems reasonable to postulate that the sudden deaths which occurred in these families have been arrhythmic in nature also because all these cases were clinically affected by ACM. This is consistent with the fact that most of probands and affected family members with *CDH2* pathogenic/likely pathogenic variants, had an arrhythmic phenotype

(20/24, 83%), while the evolution toward heart failure appeared to be rare despite adequate follow-up.

These observations matter in terms of risk stratification and clinical management. Accordingly, *CDH2* genetic screening should be considered in patients with a definite diagnosis of ACM but without significant variants in major desmosomal genes. The identification of the disease-causing variant has clinical consequences for probands and family members alike. Given that the disease progression and arrhythmic risk for ACM patients are mostly linked to physical activity,[1,5] the early identification of family members with subclinical or silent forms is important in order to establish preventive strategies that might have a favorable impact on the natural history of the disease.

Another issue to be considered is that, if the concept that the *CDH2*-associated ACM has a predominantly arrhythmogenic profile will be validated - similar to other genetic forms of ACM, such as those linked to lamin A (*LMNA)*, filamin C *(FLNC)* and phospholamban (*PLN)*[4] - specific recommendations for ICD implantation might ensue.[25] Clearly, all these observations are based on a limited number of patients. Further research and wider ascertainment strategies should provide more robust data for accurate genetic counseling of patients with *CDH2* variants.

Mechanistic implications of CDH2 variants and electrical impairment in ACM

At present, the exact mechanism by which *CDH2* variants may affect cadherin 2 protein function and foster the clinical development of ACM is unknown; however, current knowledge allows discussing possible hypotheses.

The only non-missense *CDH2* variant identified in this study, the p.N658Kfs*3, is expected to produce an aberrant mRNA transcript likely to be degraded by nonsense-mediated RNA decay[26] without being translated into protein, leading to loss-of-function by

haploinsufficiency. The haploinsufficiency mechanism has been described previously for truncating variants in proteins primarily involved in cell adhesion, such as the desmosomal cadherin DSC2[27] and other components of the desmosome.[28,29] Cadherin 2 (or N-cadherin) haploinsufficiency, in particular, has been mimicked in vivo in a cardiac-specific mouse model (heterozygous CKO) where it decreases connexin 43 plaque size leading to an increased susceptibility to ventricular arrhythmias.[30] Connexin 43 is the main structural component of gap junctions at the intercalated disc, and its reduction results in slow conduction, favoring arrhythmias by reentry.[31] Furthermore, in these mice, also the expression of the zonula occludens 1, another protein of the intercalated disc recently implicated in ACM pathogenesis,[15] was significantly reduced,[30] potentially concurring to the destabilization of gap junctions.

CDH2-encoded cadherin 2 (N-cadherin) is part of a protein cluster at the intercalated disc that also includes the cardiac sodium channel $Na_v1.5$[32] and the ability of these two proteins to influence each other has been demonstrated in induced pluripotent stem cell-derived cardiomyocytes of an ACM patient,[33] suggesting another potential mechanism of arrhythmogenesis.

The majority of pathogenic or likely pathogenic variants identified so far in *CDH2* are missense variants. A reasonable hypothesis for their mechanism of action could be that since they all reside in the extracellular EC domains of the protein (Figure 1), these variants may adversely affect normal adhesive function. Such a mechanism was recently demonstrated in vitro for *CDH2* missense variants associated to the ACOG syndrome, a multisystemic disorder characterized by developmental delay and/or intellectual disability, ocular and genital defects and congenital heart abnormalities.[34] In this condition, mutant cadherins with missense variants in EC4-EC5 showed a defect in cell-cell adhesion, with consequences both on self-binding and trans-binding with wild type cadherin 2 proteins.[34] Another multisystemic

disorder recently associated to genetic variants in *CDH2* is the Peters anomaly, mainly characterized by corneal abnormalities.[35] The reason why some *CDH2* variants may create these multisystemic disorders while others result in a pure ACM phenotype is currently unknown; further studies are needed to unravel the functional effect of specific *CDH2* variants and the molecular mechanisms leading from cadherin 2 dysfunction to different phenotypes.

Limitations

One limitation, intrinsic to the nature of collection of data from multiple sources, is the limited number of family members that could favor an ascertainment bias towards the more severe end of the spectrum of the disease. The family members effectively evaluated (*CDH2*-variant positive, with available clinical data) are only part of all other potential carriers. The evaluation of further *CDH2*-positive patients should reflect more precisely the different phenotypic variability present in this disease subtype, and may modulate the severity of disease or the specific clinical features emerged so far.

This implies that the severity of *CDH2* variants, as well as their association with other cardiac phenotypes beyond ACM, may need to be refined once a greater number of positive patients will be available.

Conclusions

The contribution of *CDH2* clinically actionable variants to ACM is small but significant. Indeed, this rare genetic form appears to be associated in our cohort with a high arrhythmogenic profile and, therefore, its evaluation may be reasonable in patients affected by ACM in whom a disease-causing variant has not been identified. Whether or not the identification of disease-causing variants in this gene should modify the management of an

otherwise still asymptomatic patient, as already happens for other rare genetic subtypes, such as *LMNA* and *FLNC*, remains to be determined.

We recognize the inherent ascertainment biases present in this type of study; however current data suggest that there is a high incidence of ventricular arrhythmias in CDH2-ACM patients, with few evolving to heart failure.

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Tables

Table 1: Genetic variants in the *CDH2* gene identified in the ACM cohort

Genomic coordinates	Exon	Nucleotide change	Amino acid change	Conservation	Protein domain	MAF/Allele count gnomAD	Variant classification	N. of probands	N. of positive FMs
chr18:25589769	5	c.614C>T	p.P205L	High	Cadherin 1 (EC1)	0.000003984/1	Likely pathogenic	1	2
chr18:25585879	6	c.781A>G	p.N261D	Full	Cadherin1-2 (EC1-EC2) interdomain	-	Likely pathogenic	1	2
chr18:25572744	9	c.1219G>A	p.D407N	Full	Cadherin 3 (EC3)	-	Pathogenic	2	2
chr18:25565494	12	c.1973dupA	p.N658Kfs*3	NA	Cadherin 5 (EC5)	-	Likely pathogenic	1	NA
chr18:25565138	13	c.2035C>G	p.P679A	Full	Cadherin 5 (EC5)	0.000003983/1	Likely pathogenic	1	1
chr18:25565036	13	c.2137G>C	p.D713H	Medium	Cadherin 5 (EC5)	0.00006365/16	VUS	1	NA
chr18:25565006	13	c.2167G>A	p.G723S	Full	Extracellular linker to trasmembrane region	0.00003982/10	VUS	1	NA

Genomic coordinates are reported according to the GRCh37/hg19 assembly. Mutation nomenclature has been assigned according to the main transcript of the *CDH2* gene (NM_001792.4). Conservation has been assessed using the Vertebrate Multiz Alignment & Conservation (100 Species) feature track of the UCSC genome browser (https://genome.ucsc.edu): Full- 100% conserved; High- at least 95% conserved; Medium- at least 70% conserved. NA=not applicable. EC= extracellular cadherin ectodomain. Minor allele frequencies (MAF)/allele counts are reported considering the total population of the gnomAD database.[16] – denotes 0% frequency. Variant classification was made according to the ACMG guidelines.[17]

Table 2: Task Force diagnostic criteria for ACM of the 6 index cases with pathogenic/likely pathogenic variants in *CDH2*

Proband (*CDH2* variant)	Age at diagnosis	Gender	Ethnicity	Dysfunction and structural alterations		Repolarization abnormalities		Depolarization/ conduction abnormalities		Arrhythmias		Family history	
				Major	***Minor***	***Major***	***Minor***	***Major***	***Minor***	***Major***	***Minor***	***Major***	***Minor***
Proband 1 (p.P205L)	34 years	M	Han Chinese	-	RVOT PLAX 30mm, RVOT PSAX 34mm. Regional RV dyskinesia (near RVOT)	TWI V1-V4	-	-	-	-	NSVT and >20,000 PVCs per 24 hours	-	-
Proband 2 (p.N261D)	17 years	M	Caucasian	Dilatation of RV, with aneurysm in RVOT. Fibro-fatty replacement on endomyocardial biospy	-	Negative TWs V1-V3	-	-	-	LBBB sustained VT, superior axis	12,000 PVCs		
Proband 3 (p.D407N)	26 years	M	Unknown	Important dilatation of RV, hypokinetic. LVEF = 45%, hypokinetic LV	-	TWI V1-V3	-	Epsilon waves	Late potentials: QRS=143, LAS=74, RMS=3,6	Electrical storms. Sustained VT, HR= 210 bpm, LBBB pattern, axis = 0°	-	SCD at 18 years in a brother affected by ACM#	-

Proband 4 (p.D407N)	47 years	M	Caucasian	Severe impairment of RV function with mild LV dysfunction	-	TWI V1-V6	-	Epsilon waves	-	-	-	-	-
Proband 5 (p.N658Kfs*3)	59 years	F	Caucasian	Dilatation of RV 118ml/m2	-	TWI V1-V3	-	Epsilon waves	Late potentials	-	PVCs >500 per 24 hours – 4,000 PVC /24 h	-	-
Proband 6 (p.P679A)	20 years	F	Caucasian	Biventricular impairment (LVEF=44%; RVEF=34%)	-	TWI V1-V4	-	-	-	-	12,688 PVCs on Holter. Presented with cardiac syncope. ICD implanted. ICD therapy for appropriate discharge for arrhythmia cL 155 msec.	ACM in the father diagnosed via cascade screening	-

TW= T wave; TWI= T wave inversion; PVC= Premature ventricular complex; LVEF= Left ventricular ejection fraction; RVEF= Right ventricular ejection fraction; VT= Ventricular tachycardia; NSVT= Non-sustained ventricular tachycardia; LBBB=left bundle branch block "-" means not present. # Genetic testing in the 18 year old brother who died suddenly was not performed, due to lack of adequate material.

Table 3: Arrhythmogenic features of *CDH2*-related ACM patients so far described

ACM Patient	*CDH2* Mutation	Proband/FM	Sustained VT/SCD	PVCs>500/24 h/NSVT	ICD	Outcome	Source
Family 1 II-3	p.P205L	Proband	-	+ (NSVT, PVCs)	-	Alive (45 years)	This study
Family 1 I-1	p.P205L	FM - Father	-	+ (NSVT, PVCs)	-	Alive (76 years)	This study
Family 2 IV-4	p.N261D	Proband	+ (sustained VT)	+ (PVCs)	+	Alive with ICD (39 years)	This study
Family 2 IV-5	p.N261D	FM - Brother	-	+ (NSVT, PVCs)	-	Alive (36 years)	This study
Family 2 IV-6	p.N261D	FM - Cousin	-	-	-	Alive (49 years)	This study
Family 3 II-1	p.D407N	Proband	+ (sustained VT)	-	+	Alive with ICD (50 years)	This study

Family 3 II-6	p.D407N	Brother	-	+ (PVCs)	-	Alive (47 years)	This study
Family 3 III-5	p.D407N	Nephew	-	+ (PVCs)	-	Alive (16 years)	This study
Family 3 II-10	NA	FM - Brother	+ (SCD)	NA	-	SCD at 18 years	This study
Family 4 II-4	p.D407N	Proband	-	-	+	Alive with ICD (75 years)	This study
Family 5 I-2	p.N658Kfs*3	Proband	-	+ (PVCs)	-	Alive (67 years)	This study
Family 6 III-1	p.P679A	Proband	-	+ (PVCs)	+	Alive (27 years)	This study

Family 6 II-2	p.P679A	FM - Father	-	+	-	Alive (56 years)	This study
Family 7 (ACM2 III-3)	p.Q229P	Proband	+ (sustained VT, SCD)	-	-	SCD at 24 years	11
Family 7 (ACM2 III-2)	NA	FM - Brother	+ (sustained VT, SCD)	-	-	SCD at 21 years	11
Family 7 (ACM2 III-4)	p.Q229P	FM - Cousin	-	+ (NSVT, PVCs)	-	Alive	11
Family 7 (ACM2 III-5)	p.Q229P	FM - Cousin	-	+ (PVCs)	-	Alive	11
Family 7 (ACM2 II-4)	p.Q229P	FM - Aunt	-	-	-	Alive	11

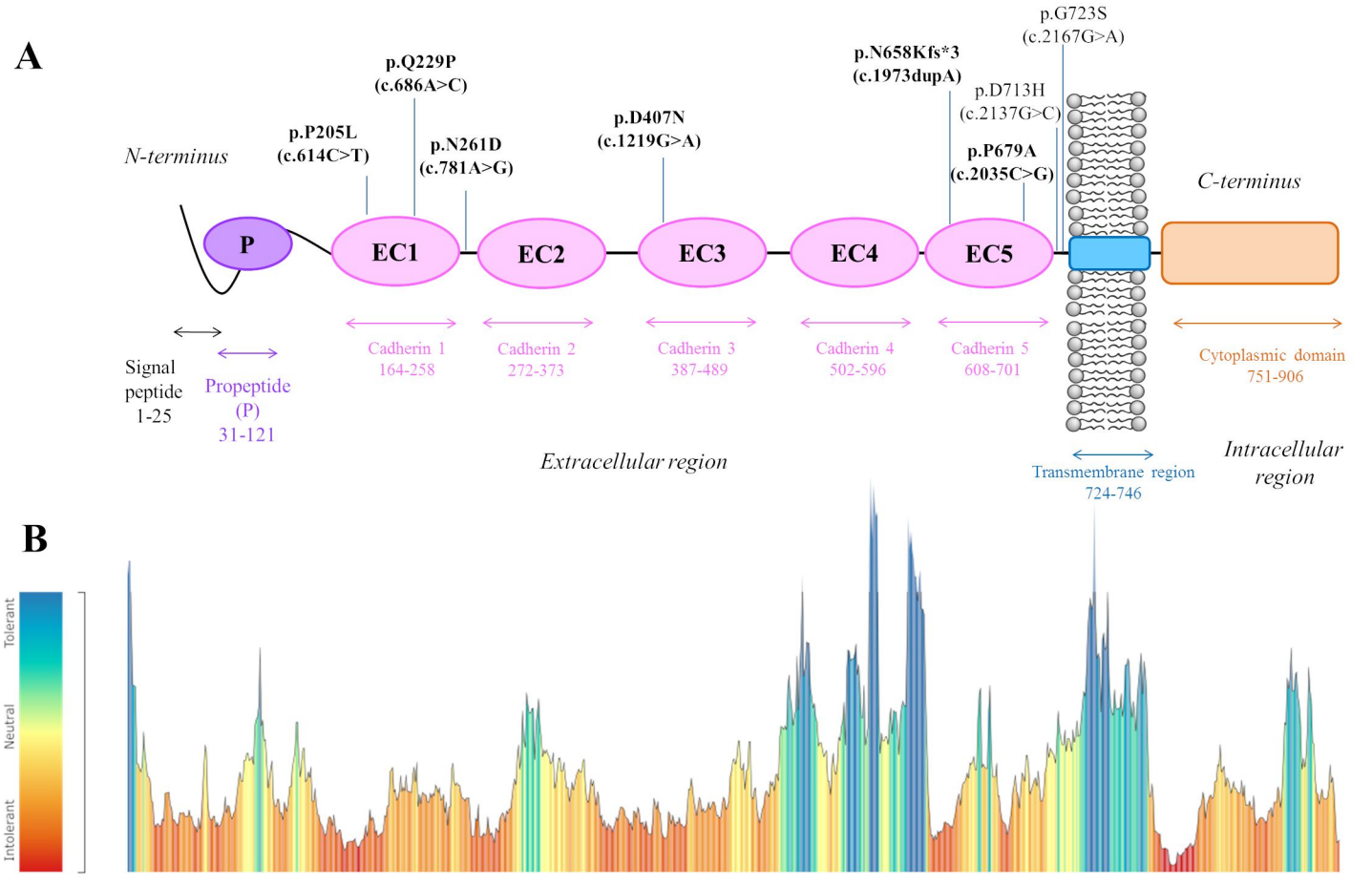
Family 7 (ACM2 II-7)	p.Q229P	FM - Aunt	-	-	-	Alive	11
Family 8 (ACM11 ACM11.2)	p.D407N	Proband	+ (sustained VT)	-	+	Alive with ICD	11
Family 9 (III.2)	p.D407N	Proband	+ (BCE with appropriate ICD shocks)	+ (NSVT, PVCs)	+	Alive (21 years)	12
Family 9 (III.1)	p.D407N	FM - Sister	-	+ (NSVT, PVCs)	+	Alive (23 years)	12
Family 9 (III.3)	p.D407N	FM - Brother	-	+ (PVCs)	-	Alive (19 years)	12
Family 9 (II.2)	p.D407N	FM - Mother	-	+ (PVCs)	-	Alive (54 years)	12

VT= Ventricular tachycardia; NSVT= Non-sustained ventricular tachycardia; SCD= sudden cardiac death; PVC= Premature ventricular complex; BCE=breakthrough cardiac event; "-" means not present; NA means not available. In "ACM patient" column, the pedigree code used in the original study was reported as well in brackets.

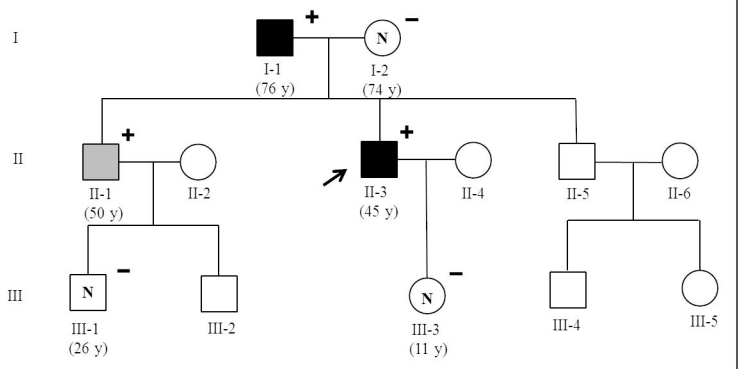
Figure Legends

Figure 1: Panel A: Schematic representation of cadherin 2 protein belonging to the superfamily of Ca^{2+} cell surface adhesion proteins. The mature protein is formed by 5 extracellular repeats (ectodomains EC1-EC5), a single-pass transmembrane region and a cytoplasmic C-terminal domain, while the N-terminal propeptide (P), following the signal peptide, is removed by proteolysis (Pfam database EMBL-EBI).[18] The length/boundaries of each domain are indicated with double-headed arrows just below the protein. All ACM-associated *CDH2* genetic variants so far identified[12,13,this study] are depicted above the protein according to their position. Mutation nomenclature has been assigned according to the *CDH2* main transcript (NM_001792.4), with the amino acid change (p.) reported first and the corresponding nucleotide change (c.) in brackets. Pathogenic/likely pathogenic variants are shown in bold. **Panel B:** *CDH2* tolerance landscape to missense variation, generated by MetaDome software analysis.[19] The tolerance score is computed by the MetaDome software using the gnomAD population,[16] as a missense over synonymous variant count ratio, calculated in a sliding window manner, for each position. The tolerance color-coded legend is reported on the left. The pathogenic/likely pathogenic missense variants identified in the ACM patient cohort all reside in red/orange regions (i.e. the most intolerant to missense variation).

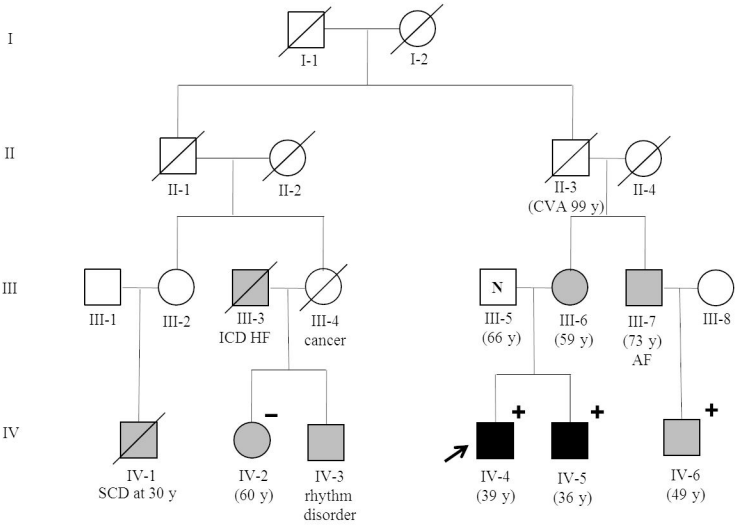
Figure 2: Pedigrees of the 6 families segregating the pathogenic/likely pathogenic variants identified in *CDH2*. The proband is indicated by an arrow. Black-filled symbols indicate affected subjects with a definite ACM diagnosis, while grey-coloured symbols stand for partially investigated subjects for whom a complete clinical picture is not available. White symbols marked up with "N" correspond to subjects with no clinical evidence of disease at latest clinical investigation. + indicates mutation-positive subjects; – indicates mutation-negative subjects. MI = myocardial infarction; PVC = premature ventricular contraction; HF = heart failure; CVA=cerebrovascular accident.


A
p.G723S
(c.2167G>A)
p.N658Kfs*3
(c.1973dupA)
p.Q229P
(c.686A>C)
p.D713H
(c.2137G>C)
p.P205L
(c.614C>T)
p.D407N
(c.1219G>A)
p.P679A
(c.2035C>G)
p.N261D
(c.781A>G)
N-terminus
C-terminus
P
EC1
EC2
EC3
EC4
EC5
Cadherin 1
164-258
Cadherin 2
272-373
Cadherin 3
387-489
Cadherin 4
502-596
Cadherin 5
608-701
Signal
peptide
1-25
Propeptide
(P)
31-121
Cytoplasmic domain
751-906
Extracellular region
Transmembrane region
724-746
Intracellular
region
B
Tolerant
Neutral
Intolerant

Family 1 (p.P205L)



Family 2 (p.N261D)



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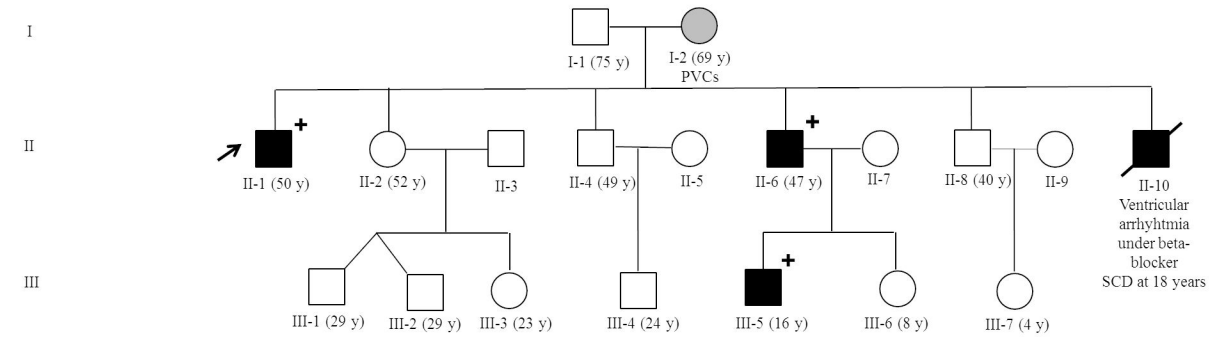

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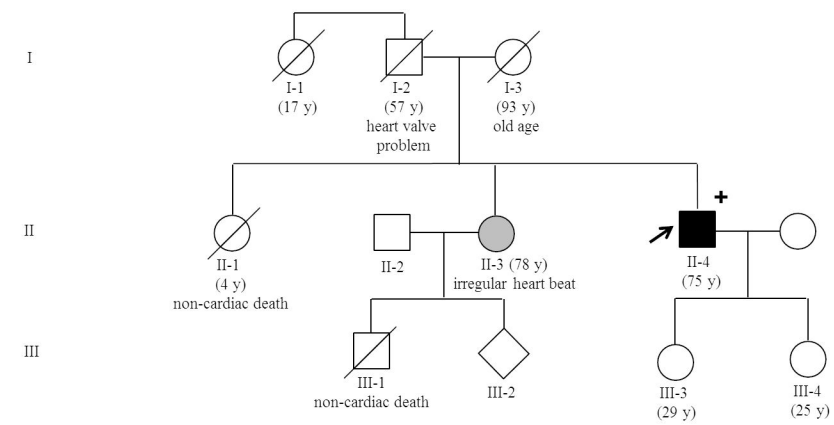
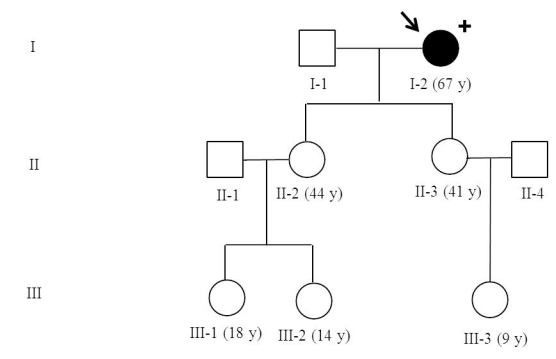


LEGEND

- Female
- Male
- Proband
- CDH2 variant present
- CDH2 variant not present
- ACM, definite diagnosis
- Partially investigated subjects, with no definite clinical classification
- no clinical evidence of disease at latest clinical investigation
- Gender not available

Family 5 (p.N658Kfs*3)



Family 6 (p.P679A)