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CLINICAL RESEARCH

Arrhythmogenic right ventricular cardiomyopathy: implications of next-generation sequencing in appropriate diagnosis

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Aims To evaluate potential differences in the genetic profile of cases with 'definite', 'borderline', and 'possible' arrhythmogenic right ventricular cardiomyopathy (ARVC) phenotype by 2010 task force criteria using a custom genetic panel after whole-exome analysis.

Methods and results We performed whole-exome sequencing in 14 cases with the clinical diagnosis ARVC using an 'Illumina HighSeq 2000' system. We presented our initial results focused on 96 known cardiomyopathy and channelopathy genes. According to the 2010 task force criteria, 7/14 cases (50%) were classified as 'definite' phenotype, 4/14 (29%) were 'borderline', and 3/14 (21%) were diagnosed with the 'possible' phenotype. Nine out of 14 patients (64%) were males, and all were Caucasians, with an average age at genetic diagnosis of 50 $\pm$ 15 years. Among the seven cases with the 'definite' phenotype, six (86%) had a putative desmosomal mutation, while none of the seven patients with a 'possible' or borderline task force classification phenotype hosted putative mutations in desmosomal genes. Four (57%) of them had rare variants in other dilated cardiomyopathy (DCM) genes.

Conclusions Most of the patients with 'definite' ARVC phenotype by task force 2010 host mutations in desmosomal genes. Weaker ARVC phenotypes host variants/mutations in other DCM genes and result in a disease spectrum, including DCM or phenocopies of ARVC.

Keywords Arrhythmogenic right ventricular cardiomyopathy • Dilated cardiomyopathy • Next-generation sequencing • Whole-exome sequencing • Cardiac channelopathies

Introduction

Arrhythmogenic right ventricular cardiomyopathy/dysplasia (ARVC) is a rare, familial heart-muscle disease that causes sudden death in young people and athletes.[1] The disease was described for the first time by Fontaine in 1978[2,3] and is characterized by either massive or partial progressive replacement of myocardium by fatty or fibrofatty tissue. This infiltration provides a substrate for ventricular dilatation and electrical instability and leads to ventricular arrhythmias, ranging from isolated premature ventricular contractions to sustained ventricular tachycardia (VT) or ventricular fibrillation.[4] The prevalence of the disease in the general population has been estimated to range from 1 in 2000 to 1 in 10 000. Eighty per cent of ARVC cases are diagnosed in patients under 40 years of age. Unfortunately, the disease is frequently only diagnosed post-mortem, particularly in young adults or athletes.[5] The disease rarely gives symptoms at young age and it is a challenge to diagnose it in early stages.

Currently, the clinical diagnosis of ARVC relies on the demonstration of structural, functional, and electrophysiological abnormalities that are caused by or reflect the underlying histological changes. Revised task force criteria were published in 2010 and classify the probability of having the disease in 'definite', 'borderline', and 'possible' diagnosis based on the following: global or regional heart

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What's new?

- Patients with 'definite' arrhythmogenic right ventricular cardiomyopathy phenotype by 2010 task force criteria host frequently desmosomal mutations.
- Patients with 'borderline' or 'possible' phenotype by task force criteria frequently host mutations in genes associated with dilated cardiomyopathy.

dysfunction, histological demonstration of fibrofatty replacement of myocardium, repolarization/depolarization abnormalities in the surface electrocardiogram, ventricular arrhythmias, family history, and genetic findings.[6]

The most common pattern of inheritance of ARVC is autosomal dominant, with variable penetrance. An autosomal recessive form has also been described on the Greek island of Naxos, where ARVC is associated with palmoplantar keratosis. In these cases, penetrance is higher than 90%.[7] Causative mutations in genes encoding desmosomal proteins have been identified in ARVC. Desmosomes are molecular complexes of cell adhesion proteins and linking proteins that attach the cell surface adhesion proteins to intracellular, cytoskeletal keratin filaments (*Figure 1*). Although ARVC is now considered to be a disorder of the cardiac desmosome,[8] some non-desmosomal genes have also been associated with the disease.[9] Despite the important knowledge generated in the last two decades, a comprehensive mutational analysis of all known ARVC genes is able to detect genetic abnormalities in only ~60% of the ARVC cases.[10] Moreover, in advanced stages of the disease, it is difficult to differentiate ARVC from idiopathic dilated cardiomyopathy (DCM). In fact, a recent study analysing the genetic basis of DCM reported mutations in desmosomal genes in 31% of the DCM-labelled cases, showing the clinical overlap of these two diseases in which the genetic diagnosis was helpful for the appropriate classification/diagnosis.[11]

During the last years, enormous technical progress has been achieved in the areas of DNA sequencing and genotyping-array technology. These technologies are leading to new strategies in the analysis of genetic diseases.[12] Next-generation sequencing (NGS) represents a cost-effective tool to perform an extensive genetic screening in a short period of time and at low costs. Next-generation sequencing technology has been approved recently by the Food and Drug Administration, which granted marketing authorization for the first high-throughput genomic sequencer in 2014.[13] Based on this technology, several comprehensive genetic panels have been developed to study genetic diseases. In the present study, we performed whole-exome sequencing (WES) in 14 ARVC cases in order to identify potential disease-causing mutations and used a panel of 96 known cardiomyopathy and channelopathy genes for filtering.

Methods

Population study

This study was conducted in full agreement with the principles of the 'Declaration of Helsinki' and laws and regulations of Switzerland. This project was approved by the Cantonal Ethical Committee of Zurich. All DNA donors available for this study signed an informed consent form approved by the Cantonal Ethical Committee of Zurich.

We performed whole-exome sequence analysis in 14 unrelated, consecutive patients enrolled in the Zurich ARVC Programme (www.arvc.ch) with ARVC diagnosis based on the task force criteria of 2010 and categorized them as definite, borderline, or possible. No family members were included.

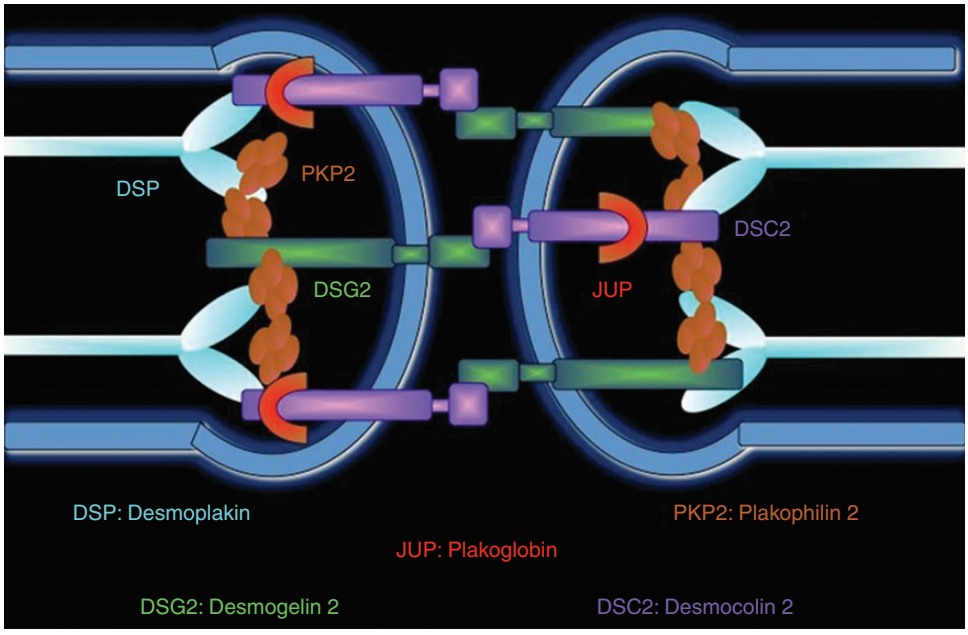

Figure 1 The desmosome and the five main proteins/genes implicated in ARVC .

Genetic data analysis

Exome sequencing was performed at Atlas Biolabs, Berlin, Germany, using the 'Illumina HighSeq 2000' platform for high-throughput sequencing. The analysis included alignment to the human reference sequence, delivering Binary Alignment/Map files and variant call format files.

From our exome data, we initially focused on a panel of 96 genes reported to be associated with cardiomyopathies or channelopathies (*Table 1*). All potential pathogenic variants, identified by exome sequencing, were confirmed by Sanger sequencing.

We used a number of different databases (HGMD Professional, ARVD/C Genetic Variants Database) and published original literature to identify known disease-causing mutations in the datasets from the patients. Novel, putative disease-associated sequence variants were distinguished from polymorphisms using the following filtering criteria: a change in the protein's primary structure, species conservation of the underlying amino acid, and an allele frequency below 1% based on the 1000 Genome Project database. For detailed sequence analysis and interpretation of sequence variations, we used the following bioinformatic algorithms and databases: variants were annotated with Alamut-Batch (including HGMD Professional) and visualized by the Alamut Viewer 2.2 (Interactive Biosoftware, Rouen, France). Additional analyses were performed with Sequence Pilot (JSI, Medical Systems, Kippenheim, Germany), SeqScape (Applied Biosystems, Rotkreuz, Switzerland), the Human Genome Browser at UCSC, and the ENSEMBL database.

We define a putative mutation of all those genetic variations rare in the population (minor allele frequency $<1\%$) and one of the following:

(1) With at least two positive computational predictions for disease association (Polyphen, SIFT, or Mutation Taster).
(2) Radical mutations.
(3) Reported as disease causing in HGMD Professional or/and http://www.arvcdatabase.info.[14]

In *TTN*, we did an exception: we reported all the rare variants but considered as putative mutations only radical mutations.

Results

In this study, we applied WES in all 14 cases and obtained high-quality WES results. On average, 87% of the bases had a coverage of $>30\times$.

All 14 unrelated patients included in this study were classified according to the ARVC task force criteria 2010 (*Tables 2* and *3*), as 'definite', 'possible', or 'borderline'.

Seven out of 14 (50%) exhibited the definite phenotype, 4/14 (29%) were borderline, and 3/14 (21%) were classified as possible phenotype. Nine patients (64%) were males and all were Caucasians, with an average age at genetic diagnosis of 50 ± 15 years (*Table 2*). The initial analysis in the 96 selected genes, previously associated with cardiomyopathies and channelopathies, showed a putative mutation in 9 out of the 14 cases (64%), which likely explains the phenotype. Three out of 14 cases (21%) exhibited digenic potential pathogenic variants/mutations (excluding *TTN* missense variants).

Among the seven cases with the definite ARVC phenotype, six (86%) had putative desmosomal mutations/variants (*Figure 2*), one carried a myosin heavy chain putative mutation (*MYH6*), and one a putative mutation in *RYR2* in addition to a putative mutation in *DSG2*-T335A. One definite case had a rare truncation in *SCN3B*, which encodes the sodium channel auxiliary β subunit $Na_V\beta3$, in addition to a novel *DSC2*-V79G putative mutation (*Table 2* and Supplementary material online, *Table S1*).

None of the seven patients with a possible or borderline phenotype according to the task force classification criteria had pathogenic mutations in desmosomal genes, only variants of uncertain significance in these genes (*Table 2* and *Figure 2*). However, four (57%) of them had rare variants in other DCM-associated genes.

In our cohort, 4 cases (29%) out of 14 carried a Titin (*TTN*) missense variant, which was the only reported genetic finding in one borderline ARVC case.

Table 1 List of genes analysed in this cohort

No.	Gene	No.	Gene	No.	Gene	No.	Gene	No.	Gene	No.	Gene	No.	Gene
1	*ABCC9*	16	*CCN2*	31	*GJA5*	46	*KCNJ2*	61	*MYPN*	76	*SCN5A*	91	*TPM1*
2	*ACTC1*	17	*CSRP3*	32	*GJD4*	47	*KCNJ5*	62	*NEBL*	77	*SGCD*	92	*TRDN*
3	*ACTN2*	18	*DES*	33	*GPD1L*	48	*KCNJ8*	63	*NEXN*	78	*SLMAP*	93	*TRPM4*
4	*AKAP9*	19	*DLG1*	34	*HCN4*	49	*KCNQ1*	64	*Nkx2-5*	79	*SNTA1*	94	*TRPM7*
5	*ANK2*	20	*DNM1L*	35	*HEY2*	50	*LAMA4*	65	*NOS1AP*	80	*SUR1*	95	*TTN*
6	*ANKRD1*	21	*DPP6*	36	*HSPB7*	51	*LDB3*	66	*PKP2*	81	*SUR2*	96	*VCL*
7	*BAG3*	22	*DSC2*	37	*JPH2*	52	*LMNA*	67	*PLN*	82	*TBX5*		
8	*CACNA1C*	23	*DSG2*	38	*JUP*	53	*MOG1*	68	*PSEN1*	83	*TCAP*		
9	*CACNA2D1*	24	*DSP*	39	*KCNA5*	54	*MYBPC3*	69	*PSEN2*	84	*TGFB3*		
10	*CACNB2b*	25	*DTNA*	40	*KCND3*	55	*MYH6*	70	*RYR2*	85	*TMEM43*		
11	*CALM1*	26	*EYA4*	41	*KCNE1*	56	*MYH7*	71	*SCN10A*	86	*TMPO*		
12	*CALR3*	27	*FGF12*	42	*KCNE2*	57	*MYL2*	72	*SCN1B*	87	*TNNC1*		
13	*CAMKII*	28	*GATA5*	43	*KCNE3*	58	*MYL3*	73	*SCN2B*	88	*TNNI3*		
14	*CASQ2*	29	*GATA6*	44	*KCNE5*	59	*MYLK2*	74	*SCN3B*	89	*TNNT2*		
15	*CAV3*	30	*GJA1*	45	*KCNH2*	60	*MYOZ2*	75	*SCN4B*	90	*TP63*		

Table 2 Summary of variants/mutations detected in this study

Case no.	Gender	ARVC task force 2010 score	Age	Reference sequence no.	Gene	Nucleotide change	Protein change	Mutation type	dbSNP132	Computational prediction			MAF (%) phenotype	Gene associated phenotype	Gen-Mut considered
										Polyphen[a]	SIFT[b]	Mutation Taster[c]			
1	M	Borderline	69	NM_032578.3	*MYPN*	c.3335C > T	p.P1112L	Missense	rs71534278	PrD	D	DC	0.2	DCM/HCM	Pathogenic
				NM_000257.2	*MYH7*	c.4423C > T	p.R1475C	Missense	rs139646545	PrD	D	DC	–	DCM/HCM	Pathogenic
2	F	Definite	42	NM_001267550.1	*TTN*	c.30274C > T	p.H10092Y	Missense	rs72650011	B	T	Pol	–	ARVC/DCM/HCM	Benign
				NM_001943.3	*DSG2*	c.152G > C	p.W51S	Missense	–	PrD	D	DC	–	ARVC	Pathogenic
3	F	Possible	23	NM_006393.2	*NEBL*	c.1775C > A	p.A592E	Missense	rs146275785	B	T	Pol	0.1	DCM	Benign
4	F	Borderline	47	NM_005751.4	*AKAP9*	c.9943A > G	p.T3315A	Missense	–	na	D	Pol	–	LQTS9/SSS	Undetermined
5	M	Definite	41	NM_002471.3	*MYH6*	c.3010G > T	p.A1004S	Missense	rs143978652	B	D	DC	0.1	HCM, DCM	Pathogenic
				NM_001267550.1	*TTN*	c.107576T > C	p.M35859T	Missense	rs72629793	B	T	DC	0.1	ARVC/DCM/HCM	Benign
6	M	Borderline	46	NM_001267550.1	*TTN*	c.102271C > T	p.R34091W	Missense	rs140319117	PrD	na	DC	–	ARVC/DCM/HCM	Undetermined
7	M	Definite	70	NM_001943.3	*DSG2*	c.1003A > G	p.T335A	Missense	rs191564913	PoD	D	Pol	0.1	ARVC	Pathogenic
				NM_001035.2	*RYR2*	c.649A > G	p.I217V	Missense	rs200642525	na	D	DC	–	CPVT/ARVC	Pathogenic
8	F	Definite	56	NM_004415.2	*DSP*	c.7999C > T	p.Q2667*	Nonsense		na	na	DC	–	ARVC	Pathogenic
9	M	Possible	45	NM_000256.3	*MYBPC3*	c.712C > T	p.R238C	Missense	–	PrD	D	DC	–	HCM, DCM, LVNC	Pathogenic
10	M	Definite	73	NM_001040151.1	*SCN3B*	c.-25-3_42del	p.?	Deletion	–	na	na	na	–	IVF, BrS, AFib	Pathogenic
				NM_004422.3	*DSC2*	c.236T > G	p.V79G	Missense	–	PrD	D	DC	–	ARVC	Pathogenic
11	M	Borderline	74	NM_000256.3	*MYBPC3*	c.2870C > G	p.T957S	Missense	rs193922380	PoD	T	Pol	–	DCM/HCM	Benign
				NM_001943.3	*DSG2*	c.877A > G	p.I293V	Missense	rs2230234	PoD	D	Pol	4.0	ARVC	Benign
12	F	Definite	52	NM_004572.3	*PKP2*	c.1378 + 1G > C	p.?	Splice Site	rs397516994	na	na	na	–	ARVC	Pathogenic
				NM_001943.3	*DSG2*	c.2137G > A	p.E713K	Missense	rs79241126	PoD	T	Pol	3.8	ARVC	Benign
13	M	Possible	30	NM_002230.2	*JUP*	c.1942G > A	p.V648I	Missense	rs143043662	B	T	DC	0.3	ARVC	Benign
				NM_001267550.1	*TTN*	c.107576T > C	p.M35859T	Missense	rs72629793	B	T	DC	0.1	ARVC/DCM/HCM	Benign
14	M	Definite	57	NM_001943.3	*DSG2*	c.473T > G	p.V158G	Missense	rs191143292	B	D	DC	0.2	ARVC	Pathogenic

All the reported variants/mutations were heterozygous.
NM, reference sequence. AFib, atrial fibrillation; ARVC, arrhythmogenic right ventricular cardiomyopathy; CPVT, catecholaminergic polymorphic ventricular tachycardia; DCM, dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy; LVNC, left ventricular non-compaction; MAF, minor allele frequency; SSS, sick sinus syndrome.
[a]Polyphen-2 (Polymorphism Phenotyping v2) prediction: PrD, probably damaging; PoD, possibly damaging; B, benign; na, not applicable; http://genetics.bwh.harvard.edu/pph2/.
[b]SIFT (Sorting Intolerant From Tolerant) prediction: D, deleterious, T, tolerated; na, not applicable; http://sift.bii.a-star.edu.sg/.
[c]Mutation Taster: DC, disease causing; Pol, polymorphism; na, not applicable; http://www.mutationtaster.org/.

Table 3 Clinical characteristics

Patient	Gender	ARVC task force 2010 score	Major criteria	Minor criteria	Depolarization abnormalities	Repolarization abnormality	Arrhythmias	RV biopsy	RV Fac	LV involvement	LVEF	ICD
1	M	Borderline	1	1	None	None	>1000 VES/24 h	NA	26	No	56	Yes
2	F	Definite	4	0	Epsilon Wave	T-wave inversion V1–3	VT LBBB superior axis	NA	27	No	57	No
3	F	Possible	0	2	Terminal activation duration >55 ms	T-wave inversion in V4	None	NA	40	No	55	No
4	F	Borderline	1	1	None	None	VT LBBB inferior axis	NA	45 (RV. Akinesia)	No	67	Yes
5	M	Definite	2	2	None	T-wave inversion in V4	>500 VES/24 h	Residual myocytes <60%	30	Yes	45	No
6	M	Borderline	3	1	Epsilon Wave	T-wave inversion with complete RBBB	VT LBBB inferior axis	NA	16	Yes	46	Yes
7	M	Definite	4	0	Epsilon Wave	T-wave inversion V1–3	VT LBBB superior axis	NA	20	No	54	Yes
8	F	Definite	3	1	Terminal activation duration >55 ms	T-wave inversion V1–3	VTs unknown origin	NA	17	Yes	26	Yes
9	M	Possible	0	2	Terminal activation duration >55 ms	None	VTs polymorphic	NA	56	No	50	No
10	M	Definite	2	1	None	T-wave inversion V1–2	VTs polymorphic	NA	14	Yes	33	Yes
11	M	Borderline	1	1	None	None	>500 VES/24 h	NA	32	No	56	Yes
12	F	Definite	4	1	Terminal activation duration >55 ms	T-wave inversion V1–3	VT LBBB superior axis	NA	32	No	65	Yes
13	M	Possible	0	2	Terminal activation duration >55 ms	T-wave inversion in V4–6	VTs polymorphic	NA	41	No	68	Yes
14	M	Definite	3	1	Epsilon Wave	None	VT LBBB inferior axis	NA	27	No	58	Yes

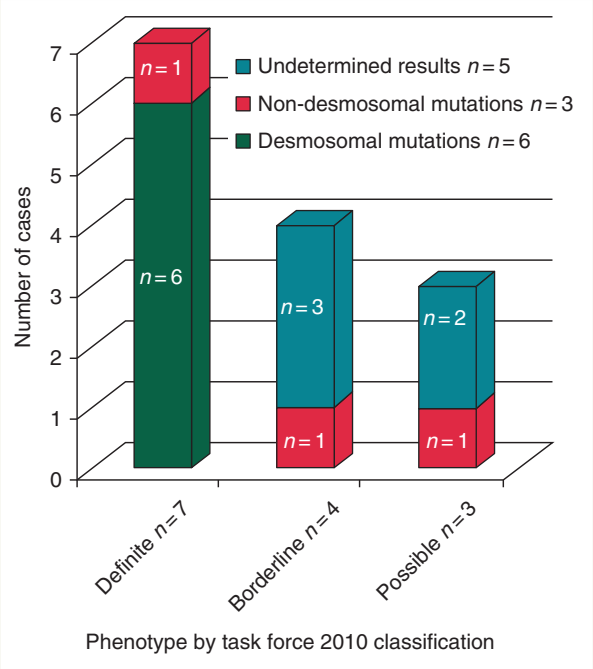

Figure 2 Phenotype–genotype correlation. The phenotype based on the task force 2010 is compared with the genetic results. The number of cases with or without desmosomal mutation is indicated. Undetermined results were those cases hosting only benign or uncertain significance variants.

Discussion

Currently, nearly 40% of the patients with an ARVC phenotype according to the task force 2010 criteria remain genetically elusive. By using WES and analysing the protein coding exons and flanking splice sites of 96 genes associated with cardiomyopathies and channelopathies, we found rare genetic variants that may explain the phenotype in 64% of the cases.

Despite the small size of our patient group, it is interesting that nearly three quarters of cases with a definite ARVC diagnosis according to the 2010 task force criteria had desmosomal mutations, while patients with a 'borderline' or 'possible' phenotype had variants/mutations in genes more often associated with DCM. It is tempting to speculate that mutations in desmosomal genes cause more often a clear ARVC definitive phenotype, while mutations in other genes could result in a disease spectrum including DCM or phenocopies of ARVC. Initially, ARVC was thought to be a disease of the desmosome; however, the description of mutations in other sarcomeric and additional proteins increased the genetic heterogeneity of ARVC and revealed an overlap with DCM.

Possibly, in some of these cases, although they fulfil the task force criteria for 'borderline' or 'possible' ARVC, the correct diagnosis is 'ARVC-like' (phenocopies) or rare forms of DCM (right-sided DCM). There is a current tendency in calling all cardiomyopathies involving the right-side 'ARVC', this is probably not correct. In terms of genetics, the ARVC-like group in this study host mutations/variants in known DCM genes. Potentially, the outcome is also different. What is the utility of distinguishing properly between ARVC from other cardiomyopathies? Patients with ARVC have more insidious ventricular arrhythmias, which usually require complex ablation procedures, and a high rate of ventricular arrhythmia recurrence is expected due to the development of novel ventricular arrhythmia circuits. The correct genetic classification is allowing better prospective studies, evaluating outcome, response to treatment, and potentially the development of specific treatment based on the genotype.[15]

One case in our cohort with the 'definite' ARVC phenotype according to the 2010 task force criteria had a *DSC2*-V79G mutation in addition to a rare and novel deletion in *SCN3B*, which encodes the sodium channel auxiliary β subunit $Na_V\beta3$. This gene had been previously associated with Brugada syndrome[16] and idiopathic ventricular fibrillation.[17] It is well known that sequence alterations in the cardiac sodium channel $Na_V1.5$, encoded by *SCN5A*, can give rise to DCM.[18] However, none of the sodium channel β subunits have been associated before with DCM or ARVC. Interestingly, there is recent evidence of a direct link between some desmosomal gene dysfunctions and reduced cardiac sodium current.[19] Thus, some ARVC cases might exhibit a reduced sodium current, which explains the overlap between ARVC and Brugada syndrome phenotypes.[19–21]

In four cases, we found rare missense *TTN* variants, encoding Titin, the largest protein in the human body, which has been possible to screen only by using NGS. Radical mutations in *TTN* (nonsense, frame shift, splice site mutations) have a higher probability to be pathogenic than missense mutations. Although we described in this article our rare findings in *TTN*, we cannot assume pathogenicity since all of these variants were missense.[22,23]

Limitations of the study

We did not perform a detailed analysis of the gene regions potentially not covered in our target genes. Exome analysis has shown to have variability of coverage within and across several heart genes. Classification of the variants as pathogenic was based on bioinformatic data and previous reports, and we cannot definitely conclude on the pathogenicity of the variants or putative mutations in the absence of co-segregation or functional studies.

Conclusion

Comprehensive genetic data are now available with the broad use of NGS for genetic diagnosis. With this diagnostic tool, we have better overview of the genetic susceptibility to arrhythmias and cardiomyopathies. The use of a specific desmosomal-ARVC screening panel would have detected only half of the variants reported here. In contrast, a broad cardiac-genetic testing panel provides a detailed overview of genetic variants that might contribute to the phenotype.

Supplementary material

Supplementary material is available at *Europace* online.

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Conflict of interest: none declared.

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