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Chapter 10

Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy. Identification of a Novel Plakophilin-2 Founder Mutation (c.2489+4A>C) in Dutch Patients

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Submitted

Abstract:

Background Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy (ARVD/C), characterized by fibrofatty replacement of cardiomyocytes in the right ventricle, is associated with life-threatening arrhythmias. Frequently a genetic predisposition is demonstrated. A novel A to C change (c.2489+4A>C) near the splice-donor site of intervening sequence 12 of the Plakophilin-2 gene was found in four separately ascertained Dutch ARVD/C families, suggesting a new founder mutation.

Methods Genealogical studies were undertaken, as well as haplotype analysis with a 10K SNP array. RT-PCR was performed in two families in order to demonstrate the presence of aberrant messenger RNA. A total of 13 individuals with this mutation had a cardiac evaluation.

Results A shared haplotype (26.1 Mb) around Plakophilin-2 was confirmed in all four families. Based on pedigree data and haplotype sharing a common ancestor should be situated more than 8 generations ago. RT-PCR demonstrated the presence of aberrant messenger RNA. Clinical manifestations ranged from severe disease to non-penetrance in elderly mutation carriers.

Conclusion A novel founder mutation in the Plakophilin-2 the gene is described. This mutation is predicted to lead to the presence of a dysfunctional Plakophilin-2 protein, whereas most truncating mutations are expected to lead to loss of protein. Mutation carriers displayed a wide range in disease severity. The clinical data underscore that Plakophilin-2 mutations alone are not sufficient to cause disease.

Introduction

Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy (ARVD/C) is a disorder characterized by progressive replacement of cardiomyocytes by fibrofatty tissue. Usually, the myocardium of the right ventricle is primarily affected. Life-threatening ventricular arrhythmias originating from the right ventricle can be an early manifestation of the disease. Clinical diagnosis depends on fulfillment of Task-Force criteria.[1]

An important genetic contribution is evident from the fact that in 30-50% of cases one or more first-degree relatives also display signs of the disease.[2,3] Nowadays, mutations in eight different genes have been reported in ARVD/C[4-13], and other loci have been implicated, indicating genetic heterogeneity. More specifically, a large majority of mutations in ARVD/C patients has been found in genes encoding different components of the cardiac desmosome

(Desmoplakin, Plakoglobin, Plakophilin-2, Desmoglein-2, and Desmocollin-2), suggesting that ARVD/C is primarily a disease of disturbed desmosomal function.[14,15] The mechanism by which mutations in these genes lead to the distinct ARVD/C phenotype remains, to a large extend, to be elucidated. Decreased desmosome numbers, structurally abnormal desmosomes, secondary effects on other intercalated disk structures, and impaired Wnt/beta-catenin signaling, may all contribute to the ARVD/C pathophysiology.[16-18] However, regardless of the demosomal gene that is mutated, translocation of Plakoglobin from the desmosome to the cytosol may be an important common step in disease causation.[17,18]

Looking at the spectrum of different mutations reported in the Plakophilin-2 gene (*PKP2*), it is evident that truncating mutations (nonsense and frameshift) mutations are most frequent.[6,19-24] The great majority of these mutations are predicted to lead to loss of Plakophilin-2 protein by mechanism called nonsense mediated messenger-RNA decay (NMD). As a general rule, introduction of a stop codon more than 50-55 nucleotides upstream of the last exon-exon boundary of any gene will lead to the breakdown of messenger-RNA in vivo, resulting in only very little translation of the abnormal truncated protein.[25] This is a strong argument for haploinsufficiency (insufficient presence of normal Plakophilin-2) as the predominant mechanism by which *PKP2* mutations predispose to disease (as opposed to a dominant negative effect due to the presence of abnormal Plakophilin-2 protein).

It has been shown that mutations in *PKP2* are frequent in ARVD/C patients in the North-Western part of Europe and the US.[6,19,20] Up to 70% *PKP2* mutations were found in case of proven familial disease in a cohort of Dutch ARVD/C patients.[20] In a collaborative effort of five university hospitals, eight different recurrent *PKP2* mutations have been found in the Dutch population, accounting for 84% (41/49) of separately ascertained *PKP2* positive ARVD/C families. For some of them a shared *PKP2* haplotype has been demonstrated [20], while other families could be traced back to a common ancestor by genealogy, sometimes as far as ten generations back. Therefore, it is likely that several different *PKP2* founder mutations segregate in the Netherlands, which may contribute to the high prevalence of pathogenic *PKP2* mutations in Dutch ARVD/C patients.

This report discusses the clinical characteristics of patients from four different families that were found to have an identical novel splice mutation (c.2489+4A>C) at the C-terminal end of the *PKP2* gene.

Patients and Methods

Clinical evaluation

All index-patients and their relatives were evaluated at the cardiology departments of University Medical Centers in Utrecht and Amsterdam. Family members were evaluated following a genetic counseling procedure and after consent had been obtained. Evaluation consisted of at least physical examination, 12-lead ECG and echocardiography. Diagnosis was based on ARVD/C Task-Force criteria[1]. The families are referred to as family A, B, C or D.

Genealogy

Since 1997 genealogical studies have been performed in newly ascertained ARVD/C cases, in order to select patients that could contribute to the finding of new ARVD/C loci, using identity by descent strategies.[26] Investigations were carried out using online community registries, starting from information on the grandparents as supplied by the index patients.

Molecular genetic analyses

DNA was isolated from peripheral lymphocytes according to standard salt extraction protocols. In index-patients the coding region of the *PKP2* gene was analyzed, including at least 30 base pairs of flanking intervening sequences. *PKP2* was analyzed using direct sequencing in families A, B and C, while family D was pre-screened with denaturing high-performance liquid chromatography. Primers used for direct sequencing were obtained from Gerull et al.[6] Direct sequencing was performed with a BigDye Terminator DNA sequencing kit (version 2.0) on a 3730 automated sequencer (ABI, Foster City, California, USA). All sequences were analyzed using SeqScape software (version 2.1.1, Applied Biosystems). Relatives of index patients were analyzed for the presence of the c.2489+4A>C mutation only.

Total RNA was isolated from fresh blood samples using Trizol reagent (Invitrogen, Carlsbad, California, USA) and subjected to random hexamer primed RT-PCR. The obtained cDNA products were amplified by nested PCR with primers specific for *PKP2* coding sequence. *PKP2* cDNA fragments were separated according to size using agarose gel electrophoresis. Both normal and aberrant fragments were gel purified and used for direct sequencing.

A 10K SNP-array (Xba131; Affymetrix, Santa Clara, California, USA) was performed, according to the manufacturer's protocol, in all patients suspected to be clinically affected, originally in pursuit of new ARVD/C loci.

The array data were converted to genotypes by the GeneChip® DNA analysis software 2.0 (GDAS 2.0; Affymetrix).[27]

Results

Clinical evaluation

All index-patients with the c.2489+4A>C mutation fulfilled the ARVD/C Task-Force criteria.[1] The index-patient from family A (patient VI-1) (Figure 1ab) (Table 1) has been known with frequent ventricular tachycardia (VT) episodes with left bundle branch block (LBBB) morphology since the age of 20, which were treated with radio-frequency (RF) ablation. At age 36 she had an out of hospital cardiac arrest (OHCA) due to ventricular fibrillation (VF) while on sotalol. Subsequently an implantable cardioverter defibrillator (ICD) was implanted, which has delivered appropriate anti-tachycardia pacing and shocks. The index-patient from family B (patient VII:4) (Figure 1ab) (Table 1) experienced a syncope at age 26 during exercise. She was admitted with

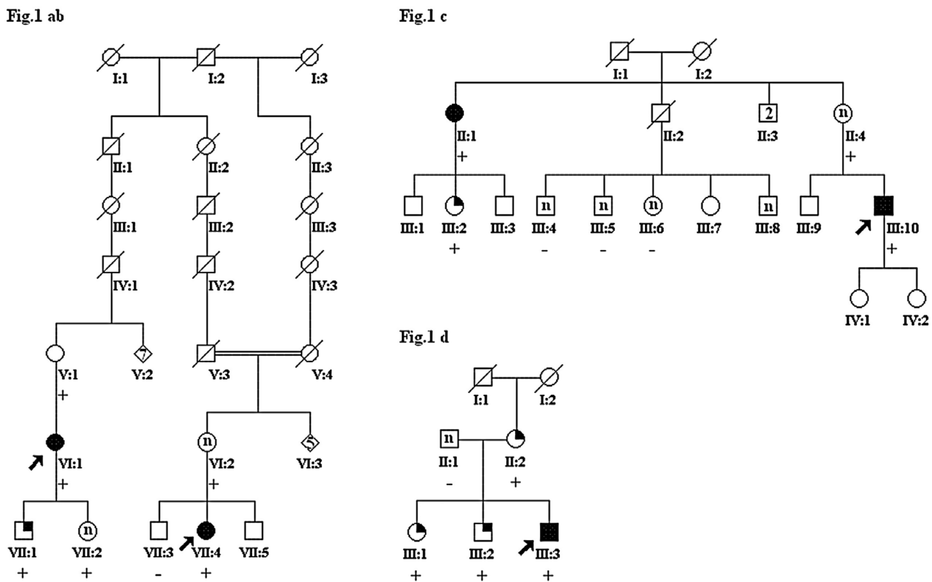

Figure 1: Pedigrees of families A, B (Figure 1ab), C (Figure 1c) and D (Figure 1d). Squares denote males, circles females. Black squares/circles denote individuals that satisfy the 1994 Task-Force criteria for a clinical diagnosis of ARVD/C (≥4 points). Upper right quadrant blackened squares/circles denote individuals that did not fulfil the Task-Force criteria, but did have abnormalities compatible with ARVD/C at their most recent cardiac examination. +/- indicates the presence or absence of the c.2489+4A>C mutation. Individuals denoted with “n” had a normal cardiac evaluation. Patient II:2 (Figure 1c) died suddenly two weeks after a myocardial infarction.

Table 1.: clinical data on *PKP2* c.2489+4A>C mutation carriers

Individual (fig.1)	Sex	Onset age (yrs)	Age first seen (yrs)	Follow-up (yrs)	Family history	Events	Epsilon wave	QRS widening [*]	Negative T-waves	LBBB VT's [†]	>1000 PVC's / 24h	RV dilation[‡]	Aneurysms[‡]	Task-Force criteria [§]	Treatment	Other
ab VI:1	F	20	20	30	index	VT VF/OHCA	+	+	V1-V5	susVT	+	+	+	6	RF-ablation sotalol / ICD	Mild progression Good LV function.
ab VII:1	M		17	7	+	none	-	-	V1	-	-	-	+	3	none	Mild progression Mild AoI.
ab VII:2	F		15	7	+	none	-	-	V1	-	-	-	-	1	none	No progression
ab VII:4	F	26	26	10	index	syncope [‼]	-	-	V1-V3	NSVT	+	+	+	4	RF-ablation [‼] none	Concurrent AVNRT No progression
ab VI:2	F		62	1	+	none	-	-	V1	-	-	-	-	1	none	Non-penetrant Limited follow-up
c III:10[#]	M	33	33	9	index	VT	-	-	V1-V2	susVT	+	-	+	5	RF-ablation disopyramide	Atypical AVNRT No progression
c II:4	F		61	0	+	none	-	-	V1	NA	NA	-	-	1	none	AF. Non-penetrant No follow-up
c II:1	F		69	7	+	none	-	-	V1-V2	NSVT	+	-	+	5	anti-coagulant therapy	Paroxysmal AF No progression
c III:2	F		35	7	+	none	-	-	V1	-	-	-	+	3	none	No progression
d III:3	M	29	29	7	index	VF/OHCA	-	-	V1-V4	susVT	-	+	+	4	ICD/ sotalol + amiodarone	BAV with mild AoI Mild progression
d II:2	F		59	7	+	none	-	-	V1	-	-	+	-	2	none	Mild progression
d III:1	F	40	33	7	+	near syncope	-	-	V1	-	-	+	+	3	ICD	Evident progression
d III:2	M	34	32	7	+	near syncope	-	-	V1-V3	NSVT	-	-	-	3	sotalol ICD	Mild progression

a wide QRS complex tachycardia (250/min). Treatment with dysopyramide was started. An AV-nodal re-entry tachycardia (AVNRT) was diagnosed at electrophysiological study (EPS), which was treated with RF-ablation, after a second arrhythmia episode. She has been free of symptoms for eight years. The index-patient from family C (patient III:10) (Figure 1c) (Table 1) was admitted to the hospital at age 33 years with sustained monomorphic VT (240/min) with a LBBB morphology during exercise. He experienced at least four other episodes of tachycardia, but no syncope. Supraventricular tachycardias also occurred, caused by AV-nodal re-entry, provoked by premature ventricular complexes. RF-ablation was performed for the ventricular arrhythmias from the right ventricular outflow tract and for the AVNRT. He has been doing well on disopyramide medication for seven years. The index-patient from family D (patient III:3) (Figure 1d) (Table 1) had an OHCA with VF at age 29. He received an ICD and was treated with metoprolol for ventricular tachycardias. At age 33 he experienced two episodes with electrical storms possibly triggered by a viral infection. Metoprolol was changed to sotalol and amiodarone was added. The patient has been doing well for three years. Clinical data on relatives carrying the c.2489+4A>C mutation are summarized in Table 1.

Genealogy

In families A and B (Figure1ab) common ancestors were found, that were born around AD 1780. Family C was originally believed to be linked to family A, however due to issues regarding uncertain paternity this could not be established. Family D could not be traced back to the same ancestors or same geographical region by genealogical studies (extending 7 generations back).

Table 1: * Positive only if QRS duration >0.12s. in V1 and <0.12s. in V6, † as recorded during exercise testing or at electrophysiological studies, ‡ as determined by echocardiography, cardiac MRI or both, § minor criteria yield 1 point and major criteria 2 points. For a definite diagnosis of ARVD/C a minimum of 4 points is required, !! syncope probably resulting from AVNRT. Thus far no symptoms have recurred after RF-ablation of AVNRT, # for this patient a biopsy was available. It showed fibrosis but no fat and was considered inconclusive. Abbreviations: AF = atrial fibrillation, AoI = aortic valve insufficiency, AVNRT = AV-nodal re-entry tachycardia, BAV = bicuspid aortic valve, F = female, LBBB = left bundle branch block morphology, M = male, NA = not available, NSVT = nonsustained ventricular tachycardia, OHCA = out of hospital cardiac arrest, PVC = premature ventricular complex, susVT = sustained ventricular tachycardia, VF = ventricular fibrillation.

Molecular genetic analyses

Analysis of the entire coding region of *PKP2*, including exon-intron boundaries, in index patients revealed a c.2489+4A>C mutation. Besides this intervening sequence mutation, no other *PKP2* alterations were found. The c.2489+4A>C mutation is still within the splice donor consensus sequence. Splice prediction software (NetGene2: www.cbs.dtu.dk/services/NetGene2/, SpliceSiteFinder: www.genet.sickkids.on.ca/~ali/ splicesitefinder.html, NNSPLICE: www.fruitfly.org/_seq_tools/splice.html, GeneSplicer: www.tigr.org/tdb/GeneSplicer/gene_spl.html, SpliceView: http://l25.itba.mi.cnr.it/~webgene/wwwspliceview.html) gave conflicting results with respect to a predicted effect on splicing (data not shown). The mutation was absent in over 150 ethnically matched controls (300 alleles). Ten relatives were also found to carry the mutation. None of the non-carriers, that had already been evaluated clinically prior to a normal DNA test result, showed any cardiac abnormalities.

RT-PCR demonstrated the presence of an abnormal *PKP2* messenger RNA (Figure 2a), indicating that the effect of the 2489+4 A>C mutation is indeed through aberrant splicing. The most abundant aberrant messenger lacks

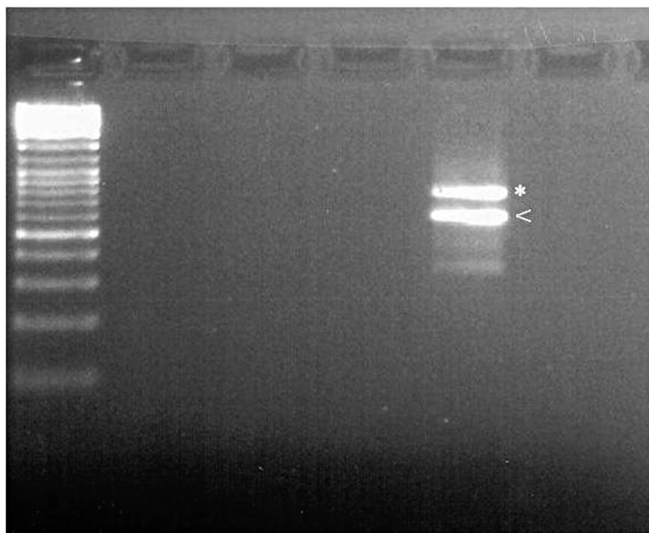

Figure 2a. Agarose gel electrophoresis showing the normal (*) and a shorter (<) cDNA product derived from mRNA of index C, together with a 100bp ladder. Nested PCR was used to amplify cDNA products. The expected normal cDNA product is 775bp in length (comprising part of exon 9, exons 10-14, and part of the 3'UTR). The shorter transcript lacks exon 12 (190bp) as shown in Figure 2b. An approximately 450bp shorter fragment is present in much lower abundance.

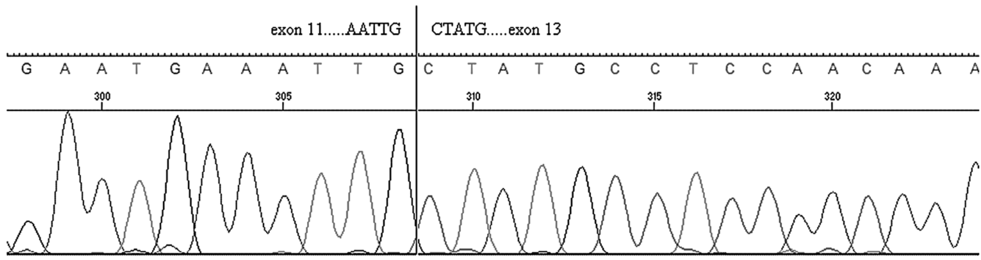

Fig. 2b: Sequencing of the cDNA derived from the abnormally spliced transcript demonstrating the skipping of exon 12. (color image: page 271)

Table 2: SNP-array data defining the shared haplotype									
SNP id	Genomic Position	Index A		Index B		Index C		Index D	
rs724903	23.213.887	B	B	B	B	A	B	B	A
rs763853	23.214.138	A	**A**	A	**A**	A	**A**	A	**A**
				26 consecutive consistent SNP's					
rs1844986	31.366.772	B	**A**	B	**A**	A	**A**	B	**A**
				***PKP2*: 32.834.954-32.941.041**					
rs2133675	33.086.481	A	**A**	A	**A**	A	**A**	A	**A**
				61 consecutive consistent SNP's					
rs1316607	49.329.157	B	**B**	A	**B**	A	**B**	B	**B**
rs1902765	50.938.088	B	A	B	B	B	A	B	B

Table 2: SNP's defining the extent of shared haplotype around *PKP2*. Haplotypes were constructed using parent-child trios. For all indexes the transmitting parent was known. The grey background identifies the mutation carrying haplotype. Shared SNP's are in boldface. Rs724903 and rs1902765 are the closest flanking SNP's that are not consistent in all four families, thus delimiting the maximum length of shared haplotype around *PKP2* at 27.7 Mb.

the entire exon 12 (190 base pairs) (Figure 2b). In the predicted protein the Ala residue at position 167 is retained but a frameshift occurs that leads to stop codon 99 residues downstream, well within the 3'untranslated region of the *PKP2* gene. Therefore, all residues encoded by exon 12, 13 and 14 are missing from the abnormal transcript, but the predicted abnormal protein

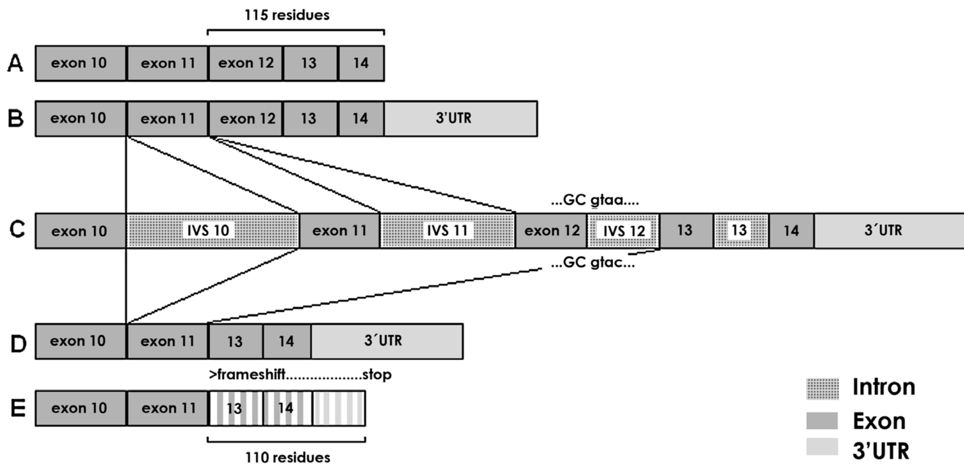

Figure 3: Aberrant splicing as a result of the c.2489+4A>C mutation. A and B represent the C-terminus of the normal Plakophilin-2 protein and the normally spliced mRNA respectively. C depicts part of the genomic DNA of the *PKP2* gene, with the position of the wild type splice site above the bar and the mutated splice site below the bar. D and E show the aberrant splice product and the predicted abnormal protein respectively. The abnormal protein is only 5 amino acid residues shorter than the wild type protein

is only 5 residues shorter than the wildtype protein (Figure 3). Since the stop codon occurs in the 3'untranslated region, the abnormal protein can not be subjected to NMD.

The SNP-array data showed a stretch of 26.1 Mb (max. 27.7 Mb) of shared haplotype (91 consecutive SNP markers corresponding to approximately 24.5 cM genetic distance) on chromosome 12p12.3-12q13.13, encompassing *PKP2* (Table 2). This was the longest stretch of shared haplotype by far, demonstrating the feasibility of a SNP array based identity by descent strategy to find disease gene loci.[27]

Discussion

PKP2 mutations are a frequent cause of ARVD/C. The *PKP2* gene was first implicated in ARVD/C through a candidate-gene approach.[6] The fact that the *PKP2* locus had not been previously found in linkage studies can be explained by low penetrance resulting in small families and misclassification of the genetic affected status. The present study in fact provides evidence for genetic linkage in retrospect as the shared mutation and SNP haplotype analysis point towards a common founder. This founder must have lived well before the 19th century. Based on pedigree data and the length of the

shared haplotype, it was estimated that the most recent common ancestor should be situated between 8 and 15 generations ago.[28] However, these are crude estimations since only four families were available. The actual age of the mutation could be considerably older. Little is known about the age of *PKP2* mutations. Our data show that mutations may have occurred many generations earlier, in absence of a family history of cardiac disease. The fact that in some families we have been able to demonstrate common ancestors as far as 10 generations back by genealogy, suggests that reproductive fitness in ARVD/C is not significantly reduced.

Although not previously described, the c.2489+4A>C mutation seems to be relatively frequent in the Netherlands, being detected in four independently ascertained ARVD/C families, so far. Another five mutations (c.235C>T, c.397C>T, c.1211-1212dupT, c.2386T>C and c.2489+1G>A) have occurred at least as frequent in the Dutch ARVD/C cohort. Haplotype analysis[20] and genealogy suggest that there also may be common founders contributing to these mutations. Although a common founder for these mutations has not been demonstrated with the same level of certainty as for the c.2489+4A>C mutation, multiple founder effects may contribute to the high prevalence of *PKP2* mutations in Dutch ARVD/C patients.

The frequent occurrence of nonsense and frameshift mutations in *PKP2* [6,19-24] is indicative that the causative mechanism at the protein level is that of loss of normal PKP2 function, as most of these mutations are predicted to lead to NMD. Gerull et al. [6], while performing Western blot analysis on cardiac tissue in a patient with a *PKP2* c.2076_2077delAA mutation, demonstrated reduced wild type Plakophilin-2, but were unable to demonstrate the predicted abnormal protein. This is in keeping with the concept of NMD in the heart. As the c.2489+4A>C mutation can not be subjected to NMD it is predicted to lead to a dysfunctional PKP2 protein, disrupting the last two conserved armadillo repeat regions and completely altering the C-terminal tail. Since the N-terminal part of the protein, that is most important for binding other desmosomal proteins and targeting Plakophilin-2 to the plasma membrane[29] is intact, it can be hypothesized that the c.2489+4A>C mutation might act as a dominant negative mutation. This could lead to more severe disease or higher disease penetrance. In contrast, the pathogenic effect of splice mutations may be ameliorated when the induced aberrant splicing is not absolute, as has been shown for the only recessive *PKP2* mutation thus far.[30] In the absence of heterozygous polymorphisms in exons 12-14 of the *PKP2* gene, the presence of a normal transcript from the diseased allele could

not be assessed. The c.2489+4A>C mutation underlines that integrity of the C-terminal part of the Plakophilin-2 protein is also required for proper function.

It can not be excluded that the c.2489+4A>C mutation is, on average, associated with a more severe or milder disease phenotype, due to the limited number of observations. However, there is little evidence to support this when comparing our cases to those from the literature.

In total 14 patients with the novel c.2489+4A>C mutation (4 males, 10 females), including index patients, were identified in the four families. Despite the overrepresentation of female mutation carriers in this study, two out of four index cases were males. Nine out of ten relatives with a mutation had a cardiac work up. Only one of nine evaluated mutation positive family members (patient II:1) (Figure 1c) (Table 1) fulfilled the task-force criteria for ARVD/C. Five additional individuals had signs of ARVD/C at cardiologic investigation, but did not satisfy the diagnostic criteria. However, they could have been diagnosed with ARVD/C if modified criteria for relatives, as proposed by Hamid et al.[31], were used.

Reduced penetrance in ARVD/C may be simulated by clinical examinations that are insufficient to detect minor symptoms in mutation carriers, or by investigations at too early an age for disease manifestations to become detectable. As others have shown[21,23,32], true non-penetrance does occur in *PKP2* positive ARVD/C. The c.2489+4A>C mutation was present in three women without any sign of ARVD/C on either ECG or echocardiography. Two of them, both over 60 years of age, could be considered true non-penetrants. In contrast, another female (patient II:2) (Figure 1d) (Table 1), who had been demonstrated to have a normal echocardiography at age 59 years, developed dilation of the right ventricle during a 7 year follow-up interval, indicating that disease progression can occur even in individuals who show no signs of disease until the 6th decade. Finally, the mutation was also present in the mother of the index patient in family A. She had experienced no symptoms of ARVD/C at age 79, but had not undergone any further cardiac examinations.

Two of the mutation positive family members developed clinical complaints probably related to ARVD/C and in both of them ICD's were implanted during their follow-up periods. A 34 year old male (patient III:2) (Figure 1d) (Table 1), who had had no complaints at initial evaluation, received an ICD after he suffered a near syncope while already taking sotalol. Non-sustained polymorphic VT's of insufficient duration to warrant ICD therapy were

subsequently recorded. His sister (patient III:1) (Figure 1d) (Table 1) received an ICD shortly after she experienced a near syncope during a stressful event. Although without any signs of disease at initial evaluation at age 33, she developed aneurysmatic changes of the right ventricle in the course of 7 years.

Which preventive treatment modalities are justified in asymptomatic *PKP2* mutation carriers is currently undetermined. We suggest that they be followed up regularly in order to assess disease progression with annual echocardiography, Holter monitoring and exercise testing. Besides, additional cardiac MRI at intervals no longer than 5 years (or before implantation of an ICD) is deemed appropriate in the Netherlands.

Based on the SNP-array data we could consider the four families in this report as separately ascertained branches of a single family (same mutation on a shared haplotype), making this the largest family study reported in *PKP2* positive ARVD/C. Given the wide range in disease severity within this extended family, and other families reported, it can be predicted that genotype-phenotype studies focusing solely on *PKP2* genotype are going to be disappointing. Although we find molecular diagnosis in ARVD/C a useful tool in identifying persons at risk for developing ARVD/C, its value is still limited by the inability to accurately predict phenotype from genotype. The fact that many identified *PKP2* mutation carriers will never experience a life-threatening arrhythmia, obviates the need for identifying other genetic or environmental factors that play a role in disease causation.

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