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Report

Mutation in Human Desmoplakin Domain Binding to Plakoglobin Causes a Dominant Form of Arrhythmogenic Right Ventricular Cardiomyopathy

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Arrhythmogenic right ventricular cardiomyopathy (ARVD/C) is a genetically heterogeneous disease characterized by progressive degeneration of the right ventricular myocardium and increased risk of sudden death. Here, we report on a genome scan in one Italian family in which the disease appeared unlinked to any of the six different ARVD loci reported so far; we identify a mutation (S299R) in exon 7 of desmoplakin (DSP), which modifies a putative phosphorylation site in the N-terminal domain binding plakoglobin. It is interesting that a nonsense DSP mutation was reported elsewhere in the literature, inherited as a recessive trait and causing a biventricular dilative cardiomyopathy associated with palmoplantar keratoderma and woolly hairs. Therefore, different DSP mutations might produce different clinical phenotypes, with different modes of inheritance.

Arrhythmogenic right ventricular cardiomyopathy (ARVD/C) is a progressive disease characterized by degeneration of right ventricular myocardium, followed by fibrous-fatty replacement (Thiene et al. 1988). Myocardial degeneration may extend to left ventricle (Gallo et al. 1992; Pinamonti et al. 1992; Basso et al. 1996) and, less frequently, to interventricular septum (Basso et al. 1996). ARVD/C is usually clinically present with arrhythmias of right ventricular origin, ranging from isolated premature ventricular beats to sustained ventricular tachycardia or to ventricular fibrillation leading to sudden death (Marcus et al. 1982). ARVD/C is the most common cause of sudden cardiac death in juveniles in northeast Italy (Corrado et al. 1990).

Seven dominant forms of ARVD/C were identified so far: ARVD1 (14q24.3) (Rampazzo et al. 1994 [MIM 107970]), ARVD2 (1q42) (Rampazzo et al. 1995 [MIM 600996]), ARVD3 (14q11-q12) (Severini et al. 1996 [MIM 602086]), ARVD4 (2q32) (Rampazzo et al. 1997 [MIM 602087]), ARVD5 (3p23) (Ahmad et al. 1998), ARVD6 (10p12-p14) (Li et al. 2000 [MIM 602087]), and ARVD7 (10q22) (Melberg et al. 1999). Among them, only the gene involved in ARVD2 has been identified to date (Tiso et al. 2001). The only known autosomal recessive form of ARVD/C is associated with palmoplantar keratoderma and peculiar woolly hairs (Naxos syndrome [MIM 601214]), caused by a homozygous 2-nucleotide deletion in the plakoglobin (JUP) gene, the product of which is a key component of desmosomes and adherens junctions (McKoy et al. 2000).

Here, we report on the identification of a causative mutation in one family, in which disease was inherited unlinked to any of the different ARVD/C loci reported so far.

The index case of the present study (subject III,9) was a young male who, at 18 years and during physical effort, experienced a cardiac arrest due to ventricular fibrillation. By thorough clinical investigation, he was diagnosed as affected with ARVD/C. His family included 26 members in four generations. All family members gave informed consent to be evaluated and to give blood samples for DNA study. The protocol (Nava el al. 2000) included: 12-lead electrocardiogram (ECG), signal-averaged ECG, 24 h Holter ECG, 2D-echocardiogram. Ad-

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Table 1

Clinical Data for Family Members Fulfilling Diagnostic Criteria for ARVC/D, According to McKenna et al. (1994)

Patient	Result for Criterion							No. of criteria (major/minor)
	Familial History (Minor)	Repolarization abnormalities (Minor)	Depolarization/conduction abnormalities		Right ventricular kinetic alterations		Type of ventricular arrhythmias[a] (Minor)	
			Major	Minor	Major	Minor		
II,4	+	+	+	+	+	+	Sustained VT	2/4
II,6	+	+	+	+	+	−	Sustained VT	2/3
III,4	+	+	−	+	−	+	−	0/4
III,9	+	+	+	+	+	−	VF, PVCs	2/3
III,10	+	+	−	+	−	+	PVCs	0/5
III,12	+	+	−	+	−	+	−	0/4
III,13	+	+	−	+	−	+	PVCs	0/5
IV,3	+	+	+	−	−	−	VF	1/2
IV,4	+	+	−	−	−	+	PVCs	0/4
IV,6	+	+	−	−	+	−	PVCs	1/3
IV,9	+	+	−	−	+	−	−	1/2

Note.—A plus sign (+) indicates presence, and a minus sign (−) indicates absence.

[a] VF = ventricular fibrillation.

ditional procedures (right and left ventricular angiography with coronary arteriography) were performed only on strict clinical indication. Diagnosis was made according to McKenna et al. (1994): affection status is established when two major criteria, one major criterion plus two minor criteria, or four minor criteria are fulfilled. Criteria involve genetic, structural, histological, electrocardiographic, and arrhythmological aspects. After clinical evaluation, 11 individuals were identified as being affected with ARVD/C (table 1) on the basis of ECG, echocardiographic, and/or angiographic findings; ventricular arrhythmias with left bundle branch block morphology; and/or presence of late potentials at signal averaged ECG (McKenna et al. 1994; Nava et al. 2000). Typical ECG and echocardiographical images are shown in fig. 1. Three patients (II,4; II,6; and III,9) manifested a severe form of the disease, three (III,13; IV,3; and IV,6) a moderate form, and the remaining ones displayed a mild form. In this last group of patients, repolarization abnormalities were present at ECG in all cases, accompanied, in two subjects, by isolated PVCs. Ventricular arrhythmias occurred isolated or in couplets/triplets in five patients (III,9; III,10; III,13; IV,4; and IV,6), whereas, in two, they progressed to sustained ventricular tachycardia (II,4 and II,6). In all individuals, ventricular arrhythmias were characterized by left bundle branch block morphology, with left axis deviation in six cases, and with right axis deviation in one. Two patients ex-

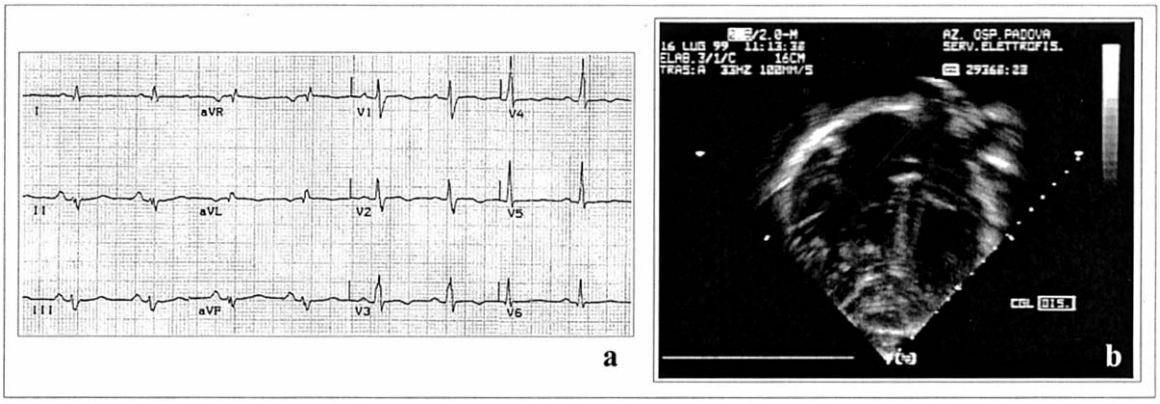

Figure 1 *a,* Twelve-lead ECG of patient III,9 (index case); presence of low voltages of QRS, QRS duration in V1-V2>V5-V6, negative T wave from V1 to V5. *b,* 2D-echocardiogram of the same patient (apical four-chamber view). Right ventricle (*left of panel*) is severely dilated, with trabecular disarrangement; left ventricle also appears to be affected.

Table 2

Two-Point LOD Scores for Different Recombination Fractions for 6p24 Markers in the Family Reported in Figure 2

	LOD Score at θ =								
Marker	.00	.01	.05	.10	.20	.30	.40	θ_{max}	Z_{max}
D6S1574	−1.60	1.69	2.15	2.13	1.75	1.18	.52	.05	2.15
D6S1640	3.95	3.88	3.58	3.20	2.38	1.49	.57	.00	3.95
D6S309	4.32	4.25	3.95	3.57	2.74	1.82	.83	.00	4.32
D6S470	4.27	4.20	3.91	3.53	2.71	1.80	.82	.00	4.27
D6S1721	3.64	3.58	3.32	2.99	2.28	1.50	.66	.00	3.64
D6S259	−4.25	−2.28	−.82	−.23	.20	.27	.17	.30	.27

Note.—LOD scores were calculated by assuming 0.95 penetrance as restrictive criterion.

perienced ventricular fibrillation that, in one case, led to sudden death (IV, 3). During follow-up, which lasted, depending on the patient, for 2–17 years (average 10 years), three individuals (II,4; II,6; and III,9) showed a progression of the disease with left ventricular involvement. In these patients, right ventricle appeared severely dilated at 2D-echo (mean value of right ventricular and diastolic volume = 130 ± 10ml/m2), and ratio between right and left ventricular volumes was higher than 1.7. The left ventricular kinetic alterations were localized in the posterior wall in two cases and in the anterior wall and apex in the third case. Ejection fraction appeared more severely depressed (33 ± 2) in the right ventricle than in the left (45 ± 5).

Two patients died suddenly (II,1 and IV,3) at ages 65 years and 15 years, respectively, and another (II,6) died because of heart failure at age 68 years. No affected subjects in the family showed phenotypic peculiarities such as woolly hairs, skin alterations, or additional genetic diseases.

Genetic investigation revealed no linkage of the disease running in the family with any DNA marker of known ARVD loci. For this reason, a 5-cM genome-wide scan was performed to map the disease locus. A genome scan was performed using 700 microsatellite markers (Applied Biosystems Prism Linkage Mapping Set Version 2.5). All family members were additionally genotyped for D6S1640, obtained from MWG Biotech. In addition, for assessing marker order and intermarker distances on chromosome 6, data from the Human Genome Browser (April 2002 release) was used.

Significantly positive linkage was detected for D6S1640, D6S309, D6S470, and D6S1721 (Z_{max} = 4.32 at $\theta = 0$ for marker D6S309), mapped to 6p24 (table 2). All patients shared a common haplotype (fig. 2). Since the novel locus (6p24) described in this paper is the eighth reported so far for dominant ARVDs, it should be named ARVD8. Penetrance, estimated in the former two generations of the family, was ~50%. ARVD8 is probably infrequent, at least in northeast Italy: among 16 families in which we have firmly established linkage with ARVD/C loci, the family reported here is the only one in which the disease was linked to 6p.

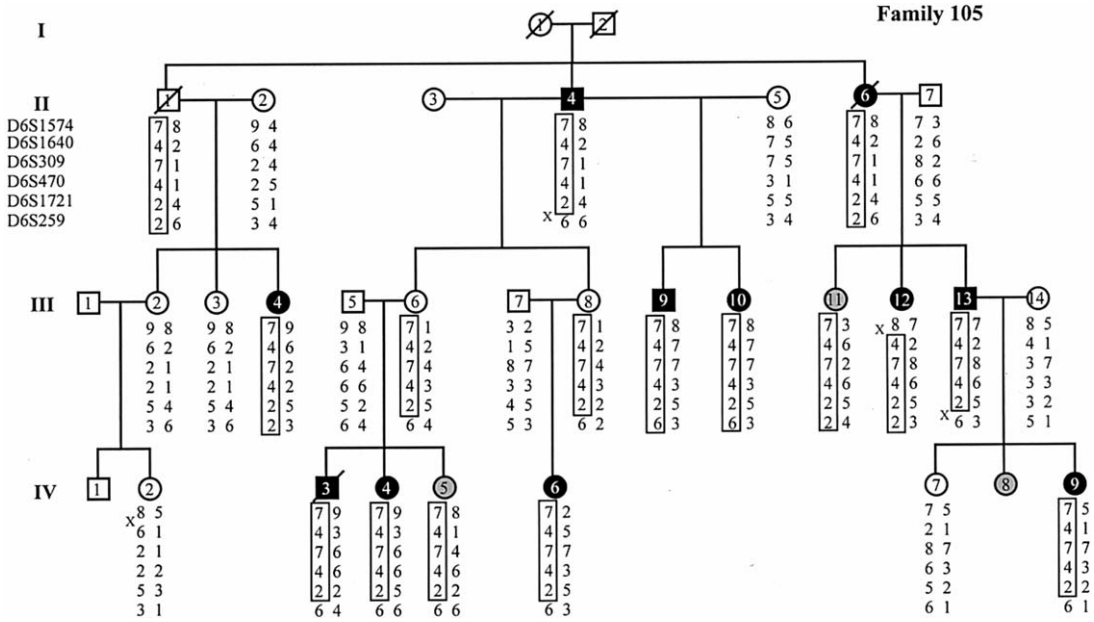

Figure 2 Family tree, showing haplotypes for chromosome 6p24 markers (ordered from telomere to centromere). Filled squares or circles indicate affected individuals; shaded squares or circles indicate individuals still under investigation. The shared disease-associated haplotype is boxed. The critical region, identified by informative recombination events (indicated by *X*), occurred in individuals III,12, II,4, and III,13.

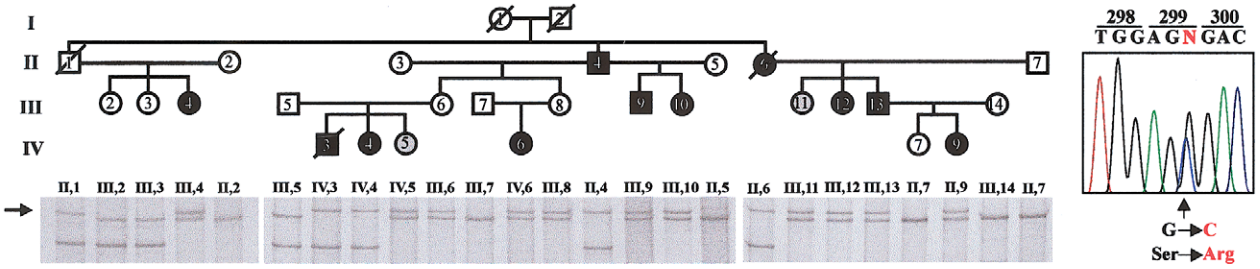

Figure 3 All investigated patients showed the presence of an additional band, visible on top of the electrophoretic pattern (*arrow*), in PCR amplicons of desmoplakin exon 7, once subjected to SSCP analysis. DNA sequencing of such amplicons revealed a C→G transition (codon 299, from AGC to AGG, resulting in a Ser→Arg substitution), which is present only in the affected members of the family. The lower band, detected by SSCP analysis in some individuals, corresponds to an additional polymorphism (I305F) identified in the same exon.

Recombinations detected in subjects III,12, II,4, and III,13 enabled us to define a critical region between D6S1574 and D6S259, spanning ~9 Mb, which was confirmed by multipoint analysis (data not shown). Gene encoding desmoplakin (DSP), located between D6S1640 and D6S309, appeared as a candidate, since a homozygous DSP nonsense mutation was reported to cause, in an Ecuadorian family, a biventricular dilative cardiomyopathy associated with keratoderma and woolly hairs (Carvajal-Huerta 1998; Norgett et al. 2000), now referred as "Carvajal syndrome" (MIM 605676).

Mutation screening was performed by direct sequencing. Flanking intronic sequences of each DSP exon were determined by comparing cDNA sequence (GenBank accession number M77830) with genomic sequence, available from public databases. PCR primers flanking each exon of the human DSP gene were designed by PRIMER3. All primer sequences are available at our Web site ARVDnet. Each exon was amplified from patient genomic DNA, purified (PCR Product Presequencing kit; USB), and sequenced using the BIG DYE dideoxy-terminator chemistry (Perkin Elmer) on an ABI 377 DNA sequencer.

DNA sequencing of all DSP exons in the index case revealed a missense mutation in exon 7, (C1176G; AGC→AGG). This mutation was detected in all clinically affected individuals and in some relatives whose clinical status is still unknown, since they were very recently recruited to the study. It was detected as well by SSCP analysis (fig. 3). SSCP analysis was performed under the following conditions: 5 μl of a 35-cycle PCR mixture was denatured and separated on a nondenaturing 10% polyacrylamide gel (29:1 acrylamide:bisacrylamide, 5% glicerol), in 1 × TBE buffer at room temperature. Single-strand conformations were detected by silver staining.

C1176G mutation could not be detected in any of 240 control subjects (480 alleles) from the same population, thus suggesting such nucleotide substitution is pathogenic.

Ser residue (S299R) mutated in patients was placed at the center of a coiled, charged region, separating the two short helices of DSP subdomain Z. Such a coiled

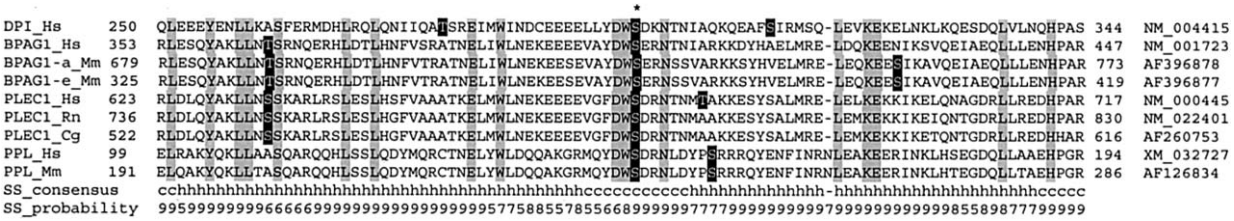

Figure 4 Alignment of the Z region from N-terminal domains of desmosomal members of the plakin family; protein names, GenBank accession numbers, and amino acid termini are reported on the right. A gray background indicates phylogenetic conservation of residues. Serine or threonine residues corresponding to putative PKC phosphorylation sites are shown in white characters on a black background. On the bottom, secondary structure (SS) consensus is shown (c = coil; h = helical) and its profile probability is reported (9, up to 100%; 8, up to 90%, and so on). The Ser residue, mutated in patients, is marked by an asterisk. This residue is central to a surface and acidic coiled region surrounded by two helices. This predicted PKC phosphorylation site is conserved in all phylogenetically related sequences: DPI (desmoplakin), BPAG1 (bullous pemphigoid antigen 1), PLEC1 (plectin), PPL (periplakin). Hs = *Homo sapiens*; Mm = *Mus musculus*; Rn = *Rattus norvegicus*; and Cg = *Cricetulus griseus*.

region is predicted to be a surface region, possibly involved in protein-protein interactions. The S299R amino acid substitution suppresses a putative phosphorylation site that is fully conserved in related proteins belonging to the same family (fig. 4), supporting the hypothesis that the amino acid change might cause a functional alteration.

DNA sequencing revealed an additional variant in exon 7: an A→T transversion resulting in the substitution of an isoleucin with a phenilalanin residue (I305F). Subsequent analysis revealed that I305F is a relatively common (~8% in frequency) polymorphism in the control population. A single-base insertion before the ATG start codon of DSP (at position –1), cosegregating with the disease in the family, was also detected. Because this insertion does not alter the optimal context of the initiation codon (Kozak et al. 1984), no pathogenic effect is expected for such a variant.

DSP, together with JUP, anchors to desmosomal cadherins, forming an ordered array of nontransmembrane proteins, which then bind to keratin intermediate filaments (IF) (Kowalczyk et al. 1997; Smith et al. 1998; Leung et al. 2002). Desmosomes are major cell-cell junctions, particularly abundant in epidermal cells and in cardiomyocytes (Gallicano et al. 1998; Smith et al. 1998). DSP consists of 2,871 amino acids, and it is predicted to be a homodimer containing two globular end domains joined by a central alpha-helical coiled-coil rod domain (O'Keefe et al. 1989; Green et al. 1990; Virata et al. 1992). A carboxy-terminal domain of DSP interacts with IF (Stappenbeck et al. 1993; Kouklis et al. 1994; Meng et al. 1997), whereas amino-terminal domain including 2-subdomain is required for DSP localization to the desmosomal plaque (Stappenbeck et al. 1993) and is binding to JUP (Kowalczyk et al. 1997).

Mutated Ser residue in the Z subdomain represents the only PKC phosphorylation site conserved in the whole N-terminal of all PKC-regulated plakins (data not shown). Such residue is likely to represent a crucial site for PKC-mediated regulation of DSP interactions with desmosomal components at the plasma membrane side. Moreover, the first 584 amino acids of the N-terminal domain, where S299R mutation was detected, are known to be involved in JUP binding and in clustering of desmosomal cadherin-JUP complexes (Kowalczyk et al. 1997).

Nonsense DSP mutations, leading to functionally null alleles, were reported to have produced striate palmoplantar keratoderma in heterozygotes, thus demonstrating that dosage of DSP is critical in maintaining epidermal integrity (Armstrong et al. 1999; Whittock et al. 1999, 2002). Conversely, heterozygotes for DSP truncated at its C-terminal domain (Carvajal syndrome) showed no keratoderma; it was suggested that, in this case, either DSP binding to IF is reduced but not lost or that loss of desmosomes-IF binding via DSP could be compensated for by other desmosomal proteins (Norgett et al. 2000).

It is possible that absence of skin defects in heterozygous carriers of DSP missense mutation S299R can be explained by considering that this mutation does not affect DSP-IF binding, which, on the contrary, is targeted by other mutations producing a keratoderma phenotype.

In heterozygotes for the S299R mutation, the majority of desmosomal cadherin-JUP complexes would be defective because of the dimeric nature of DSP functional molecules. This would explain the dominant pattern of inheritance of the disease caused by such mutation.

The involvement of two desmosomal proteins (DSP and JUP) in two different ARVD/C clinical phenotypes (ARVD8 and Naxos disease) suggests that some ARVD/C might result from defects in intercellular connections. According to present knowledge, mechanical forces applied to adherens junctions activate stretch-sensitive calcium-permeable channels via cadherins' mechanical intracellular signaling (Ko et al. 2001). Moreover, stretching of cardiomyocytes is known to modulate the elementary calcium release process from ryanodine receptor release channels (Petroff et al. 2001). Therefore, a genetically impaired response to mechanical stress might adversely affect intracellular calcium concentration and the excitation-contraction coupling. In addition, it might induce apoptosis and cellular necrosis, which, in turn, would promote fibrosis and adipose substitution, as in several muscular dystrophies. The almost selective affection of the right ventricle might be in relation to its extensibility, in comparison with that of left-ventricular free wall. It is interesting to notice that mutations in RYR2 (cardiac ryanodine receptor) cause dominant ARVD/C type 2, thus supporting the hypothesis of a key pathogenic role played by altered intracellular calcium concentration in these diseases.

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Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

ARVDnet, http://telethon.bio.unipd.it/ARVDnet/ (for list of desmoplakin primers used for amplifications and sequencing)
GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for DSP [accession no. M77830])
Human Genome Browser, http://genome.cse.ucsc.edu/index.html (April 2002 issue) (for assessing marker order and intermarker physical distances on chromosome 6)
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for ARVD1 [MIM 107970], ARVD2 [MIM 600996], ARVD3 [MIM 602086], ARVD4 [MIM 602087], ARVD6 [MIM 602087], Naxos syndrome [MIM 601214], and Carvajal syndrome [MIM 605676])
Primer3, http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi (for designing PCR primers)

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