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ARVD4, a New Locus for Arrhythmogenic Right Ventricular Cardiomyopathy, Maps to Chromosome 2 Long Arm

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Autosomal dominant arrhythmogenic right ventricular dysplasia (ARVD; MIM 107970) is a genetically heterogeneous cardiomyopathy, which often causes sudden death in juveniles and athletes. Two disease loci were previously mapped respectively to 14q23–q24 (ARVD1) and to 1q42–q43 (ARVD2). A third possible locus was assigned to 14q12–q22. We report here on a linkage study performed on three independent families with recurrence of ARVD characterized by localized involvement of the left ventricle. In these families the disease appears to be transmitted with three polymorphic DNA markers of the chromosome 2 long arm, showing a maximum lod score of 3.46 at $\theta = 0$ for the marker D2S152. The multipoint linkage analysis suggests that the novel ARVD locus, provisionally named ARVD4, maps to 2q32.1–q32.3, within the chromosomal region including markers D2S152, D2S103, and D2S389.

INTRODUCTION

Arrhythmogenic right ventricular cardiomyopathy (formerly arrhythmogenic right ventricular dysplasia, ARVD; MIM 107970) is an autosomal dominant trait with reduced penetrance (Nava *et al.*, 1988).

The disease is due to a degenerative process involving the myocardium of the right ventricle, where focal necrosis of muscle cells is followed by adipose and connective tissue replacement (Thiene *et al.*, 1988). The process is progressive and extends gradually to the whole ventricle wall, which becomes thinner than normal. The disease may run in some subjects without major symptoms. However, ventricular arrhythmias may develop, because of the electrical instability caused by the presence of these patches of adipose and connective tissue. Some arrhythmias may be very severe and eventually fatal. The most important electrocardiographic abnormalities in ARVD subjects are the T-wave inversion in the right precordial leads and the presence of late potentials in signal-averaging ECG. Diagnostic criteria were recently outlined (McKenna *et al.*, 1994). The prevalence of the disease was provisionally estimated to range from 6/10,000 in the general population to 4.4/1000 in some areas (Rampazzo *et al.*, 1994). In the past 2 years we have mapped by linkage studies two loci independently involved in the genetic determination of the disease: ARVD1 on 14q23–q24 (Rampazzo *et al.*, 1994) and ARVD2 on 1q42–q43 (Rampazzo *et al.*, 1995). The existence of a second locus (ARVD3) on chromosome 14 (14q12–q22) was suggested by a different study conducted on three small families (Severini *et al.*, 1996). We suspected further genetic heterogeneity, because among the families under investigation by our group, some showed no linkage with any of the known loci.

We report here the results of a linkage study performed on three independent families showing a recurrence of arrhythmogenic right ventricular cardiomyopathy, characterized by a localized involvement of the left ventricle.

MATERIALS AND METHODS

Clinical ascertainment. Diagnostic criteria were according to McKenna *et al.* (1994). The affected subjects of the three families considered in this study showed clinical features typical of the arrhythmogenic right ventricular cardiomyopathy. However, they showed in addition an unusual involvement of part of the left ventricle.

Family 107 was originally diagnosed in the United States and previously described by Kirsch *et al.* (1993). Two cases of juvenile sudden death occurred, which were found at the autopsy to be caused by ARVD. Two more subjects were found clinically affected with the same disease. The family shows European ancestors on both the paternal and the maternal side. No precise information on the clinical history of the ancestors is available, except that the paternal grandmother reportedly died from an unspecified cardiovascular attack. The father of the affected cases was unavailable to the clinical study, whereas the mother was found negative at the electrocardiographical examination.

Families 108 and 109, both from Northern Italy (Mantua and

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Udine, respectively), were referred to A.N. for diagnosis by local doctors. In family 108, four subjects were found to be affected after clinical examination. In the index case (II,4) the diagnosis was confirmed by angiographic study and cardiac biopsy. This subject showed ventricular arrhythmias (Lown 4b) with a left bundle branch block. Subjects I,1, II,1, and II,2 showed echocardiographic alterations and the presence of late potentials. Subjects I,1 and II,1 never complained of arrhythmias, whereas subject II,2 showed the same type of arrhythmias experienced by subject II,4.

In family 109, unrelated to the previous one, the index case (III,1) experienced a syncopal episode while dancing. He complained of cardiac arrhythmias. The electrocardiographical monitoring showed the presence of frequent arrhythmias (Lown 4b) and late potentials with a left bundle branch block. The diagnosis of ARVD was later confirmed by myocardial biopsy, echocardiography, and electrophysiological analysis. In the same family, one brother (III,2), though still asymptomatic, showed echocardiographic alterations typical of ARVD and the presence of late potentials. A similar situation was found in their father (II,2). The grandfather had reportedly died suddenly at the age of 51.

Genetic marker analysis. Blood samples were collected from affected and unaffected subjects of the involved families upon informed consent. Genomic DNA was extracted according to the salting out procedure. DNA samples were analyzed by polymerase chain reaction (PCR) using markers obtained from the Généthon list of microsatellites (Gyapay *et al.,* 1994). The polymerase chain reactions were performed as follows: 50 ng of genomic DNA was used as template with 10 p*M* each oligonucleotide primer, 0.4 units of *Taq* polymerase, 67 m*M* Tris–HCl (pH 8.8), 16.6 m*M* ammonium sulfate, 1.5 m*M* $MgCl_2$, 0.01% Tween 20, and dGTP, dATP, dTTP, and dCTP each at 100 μM in a final reaction volume of 12 μl. The samples were overlayed with 15 μl of mineral oil to prevent evaporation. PCR were performed in an MJ Research microplate thermocycler under the following conditions: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The cycle was repeated 30 times. After PCR, 2-μl aliquots of the reaction mixture were denatured and separated on a 9% denaturing polyacrylamide gel. The gels were then silver stained and dried.

Linkage analysis. Two-point linkage analysis was performed by the program MLINK of the LINKAGE software (Version 5.2) (Ott, 1989). The inheritance of an autosomal dominant trait with reduced penetrance was assumed, taking into account that this genetic model is typical of other ARVD types (Rampazzo *et al.,* 1994, 1995). According to the method adopted in our previous linkage studies, the penetrance was assumed to be 95%. However, to assess the stability of the lod score values, the z_{max} values were recalculated for different values of penetrance, from 100 to 90%. For simplicity, age-related penetrance was not included in the genetic model. In general, allele frequencies reported in the databases were used. When not available, equal frequencies of all the multiple alleles were assumed.

For the multipoint analyses the program FASTLINK (Cottingham *et al.,* 1993; Schaffer *et al.,* 1994) was used, run on a SUN (SPARC 10) computer. Simulation of linkage was obtained by S-LINK (Ott, 1989).

Information on the location of the different markers and genes on the map of the chromosome 2 was obtained from LDB (Location Data Base, University of Southampton, UK), GDB, and OMIM.

Radiation hybrid mapping. Radiation hybrid (RH) mapping was performed by the GeneBridge4 whole-genome Radiation Hybrid Panel (Research Genetics) consisting of 93 genomic DNAs from the same number of human-on-hamster somatic cell lines, plus the two control DNAs (HFL donor and A23 recipient) (Walter *et al.,* 1994). Twenty nanograms of genomic DNA was used for amplification in 10 μl of PCR buffer (16.6 m*M* $(NH_4)_2SO_4$; 67 m*M* Tris–HCl, pH 8.3; 0.01% Tween 20; 1.5 m*M* $MgCl_2$) containing each of the forward and reverse primers at 800 n*M*, 0.2 units of DNA polymerase (RTB polymerase; Bioline, Italy), and each of the four dNTPs at 25 μM. The mix was overlaid with 5 μl of mineral oil.

The reactions were carried out in 96-well plates (93 GeneBridge lines, plus 3 controls) in a PTC-225 Peltier thermal cycler (MJ Research). Cycling conditions were 1 min and 15 s at 94°C, followed by 35 cycles of 15 s at 94°C, 25 s at 55°C, 30 s at 72°C, and a final extension step for 1 min and 30 s at 72°C.

PCR products were mixed with 5 μl of loading buffer (30% glycerol, 0.25% orange G, 25 m*M* EDTA) and separated on 2.5% horizontal agarose gel in TAE buffer (40 m*M* Tris–acetate and 1 m*M* EDTA) stained with ethidium bromide. The gel was electrophoresed in the same buffer at 100 V for 1 hr. The retention profiles were submitted to the Whitehead Institute/MIT Center for Genome Research (U.S.A.), where they were processed by the program RHMAPPER (http://www-genome.wi.mit.edu/ftp/pub) and placed in the Whitehead framework of markers.

RESULTS

The three ARVD families considered by this study showed, as a peculiar pathological finding, an additional localized involvement of the left ventricle, suggesting the possibility of a novel clinical entity. These families were preliminarily investigated for the possible linkage between ARVD and polymorphic markers of chromosomes 1, 2, 6, 12, 14, and 17. These autosomes were selected for the study for different reasons: partially because some of them (1 and 14) included the already reported ARVD genes and partially because they included genes like titin, nebulin, desmin, integrins, HLA, and programmed cell death genes, which could possibly be involved in the pathogenesis of the disease.

The association with chromosomes 1, 6, 12, 14, and 17 was excluded (data not shown). Positive lod scores (1.16 at $\theta = 0$) were obtained in family 107 for eight polymorphic DNA markers (D2S152, D2S103, D2S389, D2S117, D2S309, D2S1271, D2S72, and D2S2358) lying in an approximately 21-cM range on chromosome 2q21–q35. These markers identified a haplotype invariably shared by all the affected members in the family (Fig. 1). Three additional markers (D2S2157, D2S364, and D2S159), flanking this region on both sides, showed, on the contrary, negative lod scores. Markers D2S2392, D2S104, and D2S300 were uninformative (data not shown).

Positive lod scores for markers of the region 2q32.1–q32.3 were obtained also in families 108 and 109 (Table 1). The maximum cumulative lod score was 3.46 at $\theta = 0$ for the marker D2S152. Slightly lower lod scores were obtained for the markers D2S103 and D2S389, since in family 109 they were totally or partially uninformative. A significantly positive lod score (3.46 at $\theta = 0$) was obtained also for the marker D2S309, whereas for the marker D2S117 one recombinant (II,5) was found in family 108.

Since a different map position of the markers D2S389 and D2S309 could mimic an event of double recombination, these markers were remapped in our laboratory by RH mapping. The results (not shown) confirmed their relative map position, as currently reported in LDB.

Multipoint linkage analysis, performed by FASTLINK, produced evidence of significant linkage, with a lod score of 3.46, between ARVD and the markers

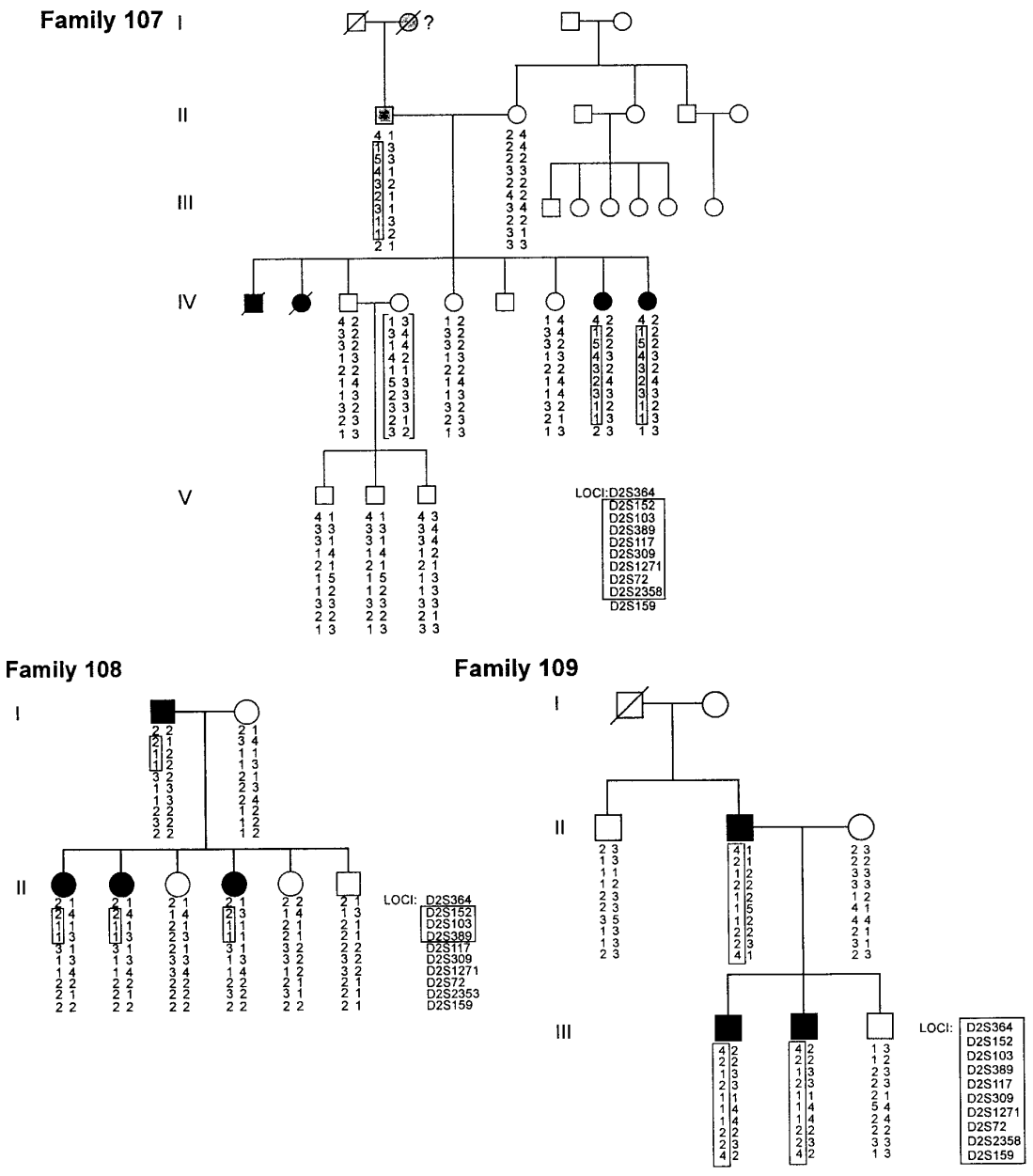

FIG. 1. Pedigrees of three independent families (107, 108, and 109) with recurrence of arrhythmogenic right ventricular cardiomyopathy. Haplotype data are shown.

D2S152, D2S103, and D2S389 (Fig. 2). The possible linkage with the above-reported markers was tested in two additional ARVD families with no involvement of the left ventricle (Nos. 101 and 103), which were described in a previous paper (Rampazzo *et al.*, 1995) and which showed negative lod scores with markers of ARVD1 (14q23–q24), ARVD2 (1q42–q43), and ARVD3 (14q12–q22). Negative lod scores were obtained in these two families for all the tested markers of the region 2q32.1–q32.3 (data not shown).

DISCUSSION

The discovery of linkage between ARVD and markers D2S152, D2S103, and D2S389 in three unrelated families suggests the existence a novel ARVD gene, provisionally named ARVD4. A significant lod score (3.46) was also obtained for the marker D2S309, but one recombinant (family 108, II,5) was detected for the marker D2S117, which maps between D2S389 and D2S309. The subject showing recombination for D2S117 was also recombinant for the marker D2S1271, which is distal to D2S309. Since the order of the markers is D2S389, D2S117, D2S309, D2S1271, as reported in LDB and confirmed by the present study for markers D2S389 and D2S309, the presence of a recombinant for D2S117 implies the existence a double crossover in a 10-cM distance.

According to LDB, the markers D2S152, D2S103, and D2S389 lie respectively at 194.301, 197.905, and

TABLE 1

Two-Point Lod Scores for Different Values of Recombination Fraction for Different Markers of the Chromosome 2q31–q35 in the Families with Recurrent ARVD Reported in Fig. 1

		Recombination fraction							
Marker		0.0	0.05	0.10	0.20	0.30	0.40	θ_{max}	z_{max}
D2S2157	Cumulative	−6.35	−0.73	−0.29	−0.02	0.03	0.01	0.30	0.03
	Family 107	−4.04	−0.23	−0.02	0.09	0.07	0.02		
	Family 108	0.00	0.00	0.00	0.00	0.00	0.00		
	Family 109	−2.31	−0.50	−0.27	−0.11	−0.04	−0.01		
D2S364	Cumulative	−3.18	0.53	0.62	0.51	0.29	0.08	0.10	0.62
	Family 107	−4.04	−0.23	−0.02	0.09	0.07	0.02		
	Family 108	0.00	0.00	0.00	0.00	0.00	0.00		
	Family 109	0.86	0.75	0.64	0.42	0.22	0.06		
D2S152	Cumulative	3.46	3.11	2.74	1.97	1.17	0.39	0.00	3.46
	Family 107	1.16	1.05	0.93	0.68	0.41	0.14		
	Family 108	1.44	1.31	1.17	0.87	0.54	0.19		
	Family 109	0.86	0.75	0.64	0.42	0.22	0.06		
D2S103	Cumulative	2.92	2.67	2.39	1.77	1.08	0.37	0.00	2.92
	Family 107	1.16	1.05	0.93	0.68	0.41	0.14		
	Family 108	1.44	1.31	1.17	0.87	0.54	0.19		
	Family 109	0.32	0.31	0.29	0.22	0.13	0.04		
D2S389	Cumulative	2.60	2.36	2.10	1.55	0.95	0.33	0.00	2.60
	Family 107	1.16	1.05	0.93	0.68	0.41	0.14		
	Family 108	1.44	1.31	1.17	0.87	0.54	0.19		
	Family 109	0.00	0.00	0.00	0.00	0.00	0.00		
D2S117	Cumulative	1.88	1.86	1.72	1.28	0.76	0.24	0.00	1.88
	Family 107	1.16	1.05	0.93	0.68	0.41	0.14		
	Family 108	0.14	1.31	0.38	0.35	0.23	0.07		
	Family 109	0.58	0.49	0.41	0.25	0.12	0.03		
D2S309	Cumulative	3.46	3.11	2.74	1.97	1.17	0.39	0.00	3.46
	Family 107	1.16	1.05	0.93	0.68	0.41	0.14		
	Family 108	1.44	1.31	1.17	0.87	0.54	0.19		
	Family 109	0.86	0.75	0.64	0.42	0.22	0.06		
D2S1271	Cumulative	2.16	2.12	1.95	1.45	0.86	0.27	0.00	2.16
	Family 107	1.16	1.05	0.93	0.68	0.41	0.14		
	Family 108	0.14	0.32	0.38	0.35	0.23	0.07		
	Family 109	0.86	0.75	0.64	0.42	0.27	0.06		
D2S72	Cumulative	1.44	1.28	1.12	0.80	0.47	0.15	0.00	1.44
	Family 107	1.16	1.05	0.93	0.68	0.41	0.14		
	Family 108	0.00	0.00	0.00	0.00	0.00	0.00		
	Family 109	0.28	0.23	0.19	0.12	0.06	0.01		
D2S2358	Cumulative	−1.95	0.04	0.43	0.25	0.43	0.15	0.10	0.43
	Family 107	1.16	1.05	0.93	0.68	0.41	0.14		
	Family 108	−3.97	−1.76	−1.14	−0.51	−0.20	−0.05		
	Family 109	0.86	0.75	0.64	0.42	0.22	0.06		
D2S159	Cumulative	−2.05	0.52	0.62	0.51	0.29	0.08	0.05	0.52
	Family 107	−2.91	−0.23	−0.02	0.09	0.07	0.02		
	Family 108	0.00	0.00	0.00	0.00	0.00	0.00		
	Family 109	0.86	0.75	0.64	0.42	0.22	0.06		

Note. Lod scores were calculated by assuming 0.95 penetrance.

199.709 cM from the top of the genetic map of the chromosome 2. This region (2q32.1–q32.3) spans about 5 cM and contains only two known genes: the collagen gene COL3A1 and the distal-less homeobox gene DLX2. However, according to OMIM, DLX2 maps to 2q32, together with CREB2 (cyclic AMP response element-binding protein-2), DLX1 (distal-less homeobox 1), INPP1 (inositol-polyphosphate 1-phosphatase), and WSS (wrinkly skin syndrome), which is supposedly due to defects of COL3A1 or COL5A2.

Distal-less homeobox genes code for transcription factors and are expressed in developing mammalian brain. Their involvement in the pathogenesis of ARVD seems improbable. Mutations of COL3A1 were reported to be associated with Ehlers–Danlos syndrome or with aortic aneurysms, but joint laxity, skin extensibility, nor aortic aneurysms were never reported in ARVD cases. Therefore, unless peculiar mutations of COL3A1 could produce a pathological phenotype with exclusive myocardial expression, this gene can hardly be considered a good candidate. On the other hand, CREB2 and INPP1 genes might represent potential candidates, since the occurrence of

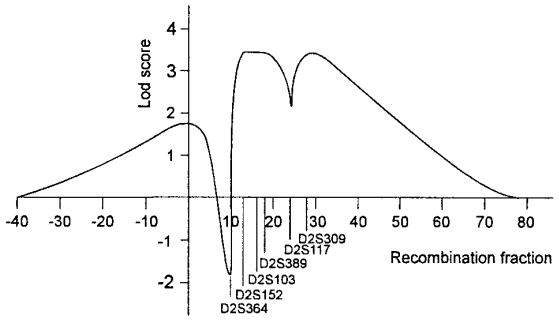

FIG. 2. Graphical output of the multipoint linkage analysis performed on the three ARVD families and involving six marker loci of the chromosome 2 long arm. For the calculations, a 95% penetrance was assumed.

apoptosis in myocardial cells of some ARVD cases was recently reported (Mallat *et al.*, 1996; Valente *et al.*, 1996).

In a slightly larger interval (between D2S152 and D2S318), including the critical region, the Human Transcript Map database reports the presence of a gene for CGRP type 1 receptor, one for the transcriptional repressor NAB1, and one for the α subunit precursor of the vitronectin receptor. The CGRP1 gene is predominantly expressed in heart and NAB1 is implicated in cell proliferation, whereas the vitronectin receptor plays an important role in the process that links cytoskeletal proteins to the extracellular matrix. Each one of these three genes might be a reasonable candidate for ARVD4.

As far as the clinical features of the disease are concerned, the affected subjects in which the disease is linked to 2q32.1–q32.3, in addition to the severe alterations of the right ventricle, show a partial involvement of the left ventricle. In principle, this should imply a more severe manifestation of the disease. This is definitely true for family 107, whereas in the other cases the progression of the disease is rather slow. It should be noticed, however, that subjects showing a severe manifestation of symptoms were invariably involved in sporting activities in their youth, whereas those with mild manifestations reported a lack of sport on regular basis. Therefore, one might suspect that the localized involvement of the left ventricle is not, per se, associated with a severe manifestation, while strenuous physical exercise seems to be dangerous for ARVD subjects, possibly because of the induced right ventricle overload.

Finally, it is worth noting that two large families with recurrence of ARVD, but with no involvement of the left ventricle (families 101 and 103), did not show linkage with markers of the region 2q32.1–q32.3, nor with any of markers associated to ARVD1 (14q23–q24), ARVD2 (1q42–q43), or ARVD3 (14q12–q22). Therefore, the existence of at least another ARVD gene, independently involved in the genetic determination of the disease, should be postulated.

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