

# Identification of mutations in the cardiac ryanodine receptor gene in families affected with arrhythmogenic right ventricular cardiomyopathy type 2 (ARVD2)

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Received 20 September 2000; Revised and Accepted 23 November 2000

**Arrhythmogenic right ventricular dysplasia type 2 (ARVD2, OMIM 600996) is an autosomal dominant cardiomyopathy, characterized by partial degeneration of the myocardium of the right ventricle, electrical instability and sudden death. The disease locus was mapped to chromosome 1q42–q43. We report here on the physical mapping of the critical ARVD2 region, exclusion of two candidate genes (actinin 2 and nidogen), elucidation of the genomic structure of the cardiac ryanodine receptor gene (*RYR2*) and identification of *RYR2* mutations in four independent families. In myocardial cells, the RyR2 protein, activated by Ca<sup>2+</sup>, induces the release of calcium from the sarcoplasmic reticulum into the cytosol. RyR2 is the cardiac counterpart of RyR1, the skeletal muscle ryanodine receptor, involved in malignant hyperthermia (MH) susceptibility and in central core disease (CCD). The RyR2 mutations detected in the present study occurred in two highly conserved regions, strictly corresponding to those where mutations causing MH or CCD are clustered in the *RYR1* gene. The detection of RyR2 mutations causing ARVD2, reported in this paper, opens the way to pre-symptomatic detection of carriers of the disease in childhood, thus enabling early monitoring and treatment.**

## INTRODUCTION

The acronym ARVD (arrhythmogenic right ventricular dysplasia) refers to a genetically heterogeneous group of cardiomyopathies characterized by progressive degeneration of the myocardium of the right ventricle, electrical instability and sudden death (1). This class of diseases, mostly inherited as autosomal dominant, is frequently involved in the cardiac

sudden deaths of juveniles and athletes (2). Several forms with dominant inheritance (ARVD1, OMIM 1907970; ARVD2, 600996; ARVD3, 602086; ARVD4, 602087; ARVD5, 604400; and ARVD6, 604401) were identified in the last few years (3–8). Arrhythmogenic right ventricular cardiomyopathy type 2 (ARVD2) is clinically different from the other forms of ARVD because of the presence of peculiar effort-induced ventricular arrhythmias, by its high penetrance and by a 1:1 male:female ratio among the affected subjects. This clinical entity was first described in 1988 by Nava *et al.* (9), when juvenile sudden death and effort-induced ventricular tachycardias were reported in a family with right ventricular cardiomyopathy. The ARVD2 disease locus was mapped to chromosome 1q42–q43 (4,10). We report here on the physical mapping of the critical ARVD2 region, including radiation hybrid placement of candidate genes, elucidation of their genomic structure and identification of cardiac ryanodine receptor (*RYR2*) missense mutations in four independent ARVD2 families. The pathogenetic role of the detected mutations is discussed.

## RESULTS AND DISCUSSION

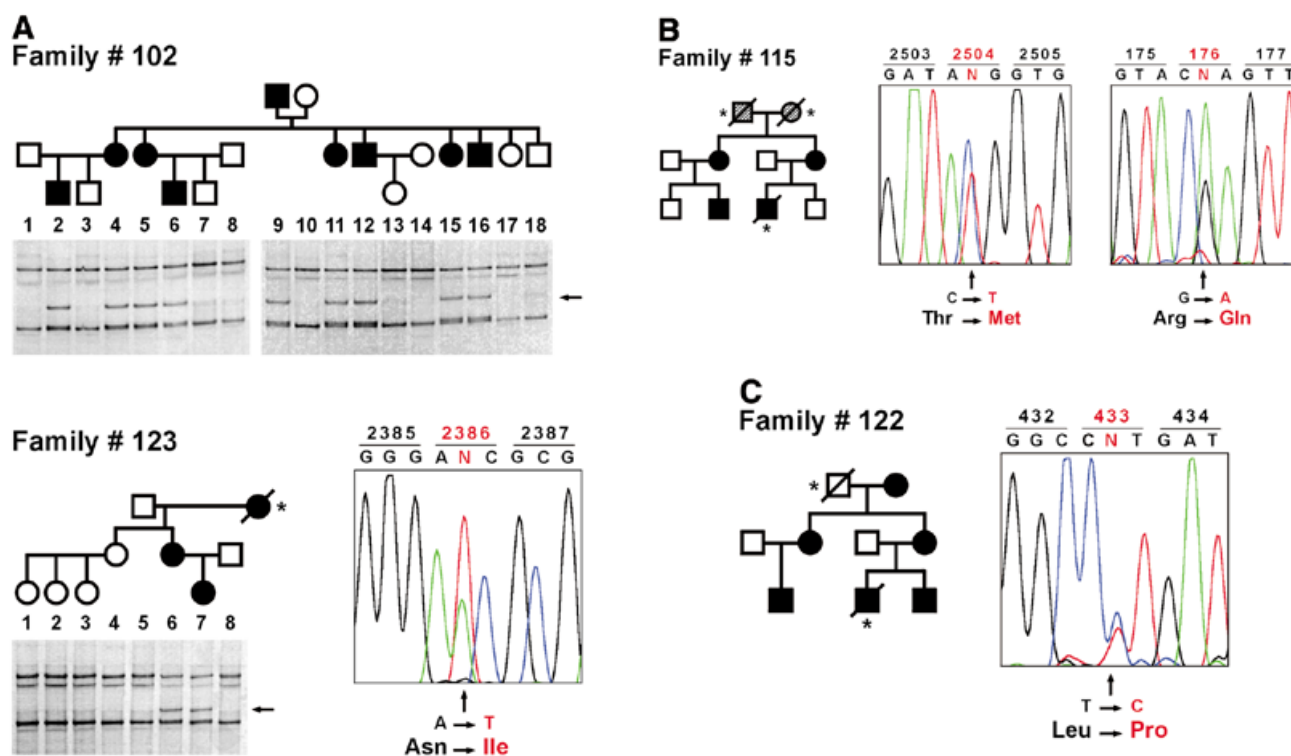
Four families showing recurrence of ARVD2 cases (Fig. 1) were detected in the course of an investigation on ARVD families, lasting over a decade. The clinical description of families 102 and 115, both from Venetia (north-east Italy), was reported elsewhere (4,10); families 122 and 123, recently recruited to the study, came from Lombardy and Venetia, respectively. In all cases, the clinical phenotype showed neither inter- nor intrafamilial variability.

The pathological trait was inherited as autosomal dominant and linkage analysis proved that the disease was inherited linked to markers *ACTN2* (CA4F/R) and *DIS2680* (data not shown).

By radiation hybrid mapping and sequence tagged site (STS) content, we mapped three ARVD2 candidate genes to the ARVD2 critical interval:  $\alpha$  actinin-2 (*ACTN2*), nidogen (*NID*)

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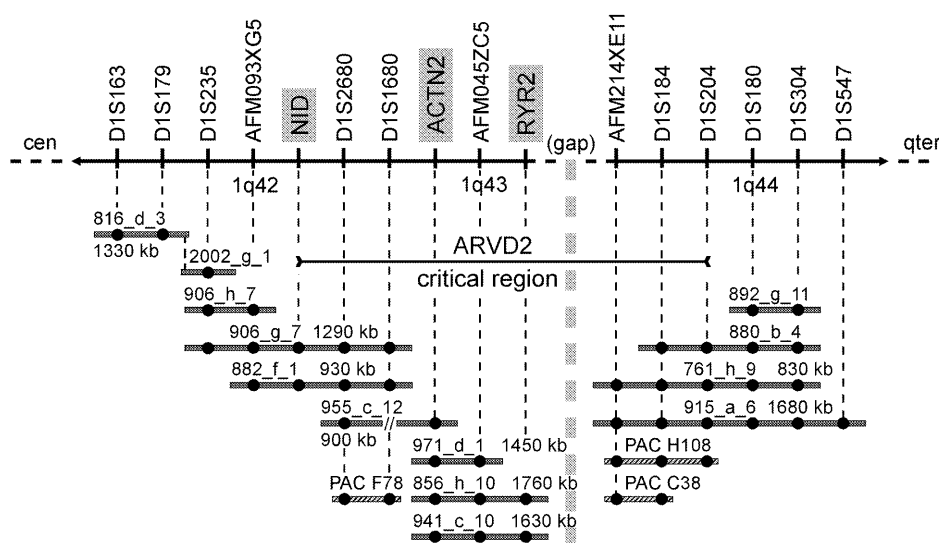


**Figure 1.** ARVD2 pedigrees and identification of non-polymorphic *RYR2* point mutations. Four pedigrees of ARVD2 families are shown, considered by the present study. The maximum cumulative lod score of 6.5 was obtained between markers *DIS2680* and *DIS184*, where *ACTN2* and *RYR2* genes are located ( $\theta = 0$ , penetrance 95%; data not shown). Asterisks indicate individuals whose DNA is not available. (A) Families 102 and 123: SSCP analysis of PCR products from *RYR2* exon 47 shows the same electrophoretic pattern in both families, with additional bands in all the affected individuals. For simplicity, only a portion of the entire pedigree of family 102 is shown. DNA sequencing of all subjects from both families reveals an A→T transversion only in all the affected members, which changes codon 2386 from AAC to ATC, resulting in an Asn→Ile substitution. (B) Family 115: DNA sequencing of *RYR2* exon 49 and exon 8 in all family members reveals two transitions (C→T in exon 49 and G→A in exon 8) only in all the affected individuals, leading to the Thr2504Met and Arg176Gln substitutions, respectively. (C) Family 122: DNA sequencing of *RYR2* exon 15 in all family members shows a T→C transition only in all patients, resulting in a Leu→Pro substitution.

and *RYR2* (Fig. 2). *ACTN2*, a cardiac-specific structural protein, anchors the myofibrillar actin filaments to the Z-disc. *NID* encodes a sulfated glycoprotein tightly associated with laminin and collagen IV, probably involved in cell–extracellular matrix interactions. RyR2 has a pivotal role in calcium release from the myocardial sarcoplasmic reticulum (SR). The genomic structure of all these three genes was determined in the course of the present work by gapped BLAST comparison of cDNA sequences with the genomic DNA sequences available in GenBank. Polymerase chain reaction (PCR) primers, designed to flank each exon, were used to screen genomic DNA from patients. Single-strand conformation polymorphism (SSCP) analysis, denaturing high performance liquid chromatography (dHPLC) and direct sequencing were used to detect point mutations. The presence of intragenic deletions was assessed by semi-quantitative PCR. The investigation on *ACTN2* and *NID* failed to detect intragenic deletions. Point mutations were detected in exons 9, 10, 12 and 21 of *ACTN2*, in exons 2, 3, 8, 9, 16, 17 and 20 of *NID* and in exons 15, 28, 37 and 59 of *RYR2*. Subsequent screening performed on a series of >200 unrelated Italian controls revealed that all the detected mutations were single nucleotide polymorphisms (SNPs). However, four RyR2 mutations (R176Q, L433P, N2386I and T2504M) (Table 1) were shown to be invariably transmitted from patient to patient along generations and were

never detected among their healthy relatives or among the control population. The N2386I mutation was detected in two families (nos 102 and 123) both from Monselice, a small town close to Padua (Fig. 1A). In these two families, SSCP analysis enabled us to demonstrate that two polymorphic amino acid substitutions in the RyR2 sequence (G1885E and Q2958R) were carried *in cis* with the presumed causative mutation and the disease, defining a rare haplotype on which the mutation occurred. The extended haplotype, defined by shared alleles at markers *DIS2680*, *DIS1680*, *ACTN2* and *DIS184*, spans at least 12 cM. This situation suggests the existence of a relatively recent common ancestor for the two apparently independent ARVD families. In family 115, two different non-polymorphic variants (R176Q and T2504M) were shown to co-segregate with the affected phenotype (Fig. 1B). Affected members of family 122 inherited the mutation L433P and the polymorphic amino acid substitution G1885E on the same allele (Fig. 1C).

The *RYR2* gene, encompassing 105 exons, encodes the 565 kDa monomer of a tetrameric structure interacting with four 12 kDa FK-506 binding proteins (FKBP12.6). Ryanodine receptor function is fundamental for intracellular calcium homeostasis and for excitation–contraction (EC) coupling (11). The RyR2 protein is thought to function by physically linking to the dihydropyridine (DHP) receptor of the t-tubule.



**Figure 2.** Genetic and physical map of the ARVD2 region. Three ARVD2 candidate genes (shaded) and 13 microsatellites from the 1q42–q44 region were ordered by combining linkage mapping data with information from radiation hybrid mapping and STS-content mapping of CEPH YACs (solid bars) and RPCI PAC clones (hatched bars). Cytogenetic localizations are based on map data from the LDB. The indicated ARVD2 critical region includes the most tightly linked markers to the disease and spans a 12 cM/12 cR/4 Mb chromosomal segment.

In myocardial cells, DHP receptor protein, activated by plasma membrane depolarization, induces a  $\text{Ca}^{2+}$  influx. The RyR2 protein, activated by  $\text{Ca}^{2+}$ , induces the release of calcium from the SR into the cytosol.

All four missense mutations detected in RyR2 in ARVD2 patients resulted in substitutions involving amino acids highly conserved through evolution (Fig. 3). In all cases, the missense mutations occurred in the cytosolic portion of the molecule. It is interesting to note that mutations detected in RyR2 by the present study cluster in two regions where mutations causing malignant hyperthermia (MH) or central core disease (CCD) are also clustered in the corresponding skeletal muscle ryanodine receptor gene (*RYR1*) (12) (Fig. 4). In particular, R176Q exactly corresponds to the RyR1 Arg163Cys mutation, for which functional studies are available (13). These mutations are believed to unblock the channel, resulting in hyperactivation/hypersensitization effects (14).

The four RyR2 mutations reported here occurred in domains of the protein which are critical for the regulation of the calcium channel. In particular, two of them (N2386I and

T2504M) are in a domain known to interact with FKBP12.6, the regulatory subunit that stabilizes the RyR2 channel (15). These mutations should make the channel more sensitive to agonists. The positive response of ARVD2 to beta-blockers, observed in our series of patients, seems to point in this direction.

Our hypothesis is that the RyR2 missense mutations might alter the ability of the calcium channel to remain closed and thus, on physical perturbation (i.e. membrane depolarization or mechanical stress), allow calcium to leak from the channel and promote a massive SR calcium release. This might be viewed as a gain of function, which is in agreement with the dominant inheritance.

Mutations enabling the RyR2 channel to open spontaneously are expected to produce a loss of EC coupling. Moreover, imbalance of the intracellular calcium homeostasis is expected to trigger apoptosis and/or cellular necrosis. Indeed, apoptosis was reported in ARVD (16,17). Impairment of intracellular  $\text{Ca}^{2+}$  release mechanism due to RyR2 mutations would probably be exacerbated by increased heart rate and by volume

**Table 1.** Non-polymorphic *RYR2* mutations detected in ARVD2 patients

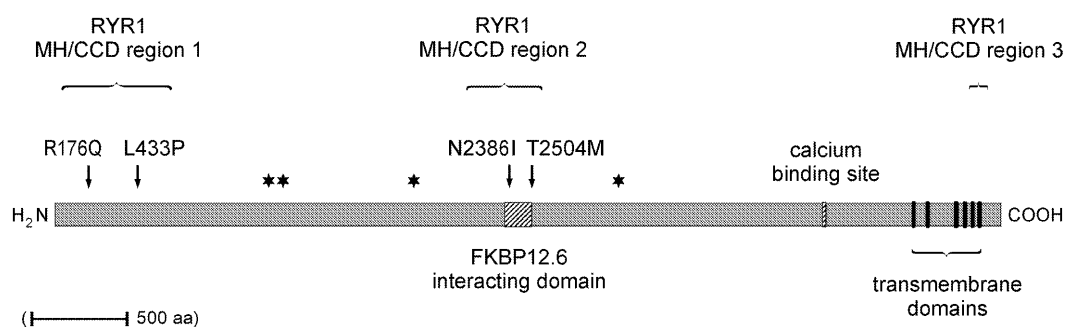
Family no.	Exon no.	Amino acid change <sup>a</sup>	Nucleotide change <sup>b</sup>	Consequence	Protein domain	Verification method
102 and 123	47	N2386I	7157A→T	Missense mutation	FKBP12.6 domain	SSCP/sequencing
115	49	T2504M	7511C→T	Missense mutation	FKBP12.6 domain	Sequencing
115	8	R176Q	527G→A	Missense mutation	Cytosolic portion	dHPLC/sequencing
122	15	L433P	1298T→C	Missense mutation	Cytosolic portion	dHPLC/sequencing

<sup>a</sup>Numbering of the amino acids refers to the RyR2 peptide sequence S72269.

<sup>b</sup>Numbering of the nucleotides refers to *RYR2* cDNA sequence NM\_001035, with the first nucleotide of ATG initiation codon as 1.

	R176Q ↓	L433P ↓	N2386I ↓	T2504M ↓
RyR2 human	RSEGEKVRVGDDLIL //	LFNRFIRGLDALSKKAK //	DDTIHMGNAIMTFYS //	RAAASLDTAALSATD
RyR2 rabbit	RSEGEKVRVGDDLIL //	LFNRFIRGLDALSKKAK //	DDTIHMGNAIMTFYA //	RAAASLDTAALSATD
RyR $\alpha$ bullfrog	RSEGEKVRIGDDLIL //	LFNRFIRGLDSLNGKNK //	ENRVHLGNAIMSFYA //	RAAASLDTAAFSTTE
RyR $\beta$ bullfrog	RSEGEKVRIGDDLIL //	LFSQFISDLDSLGNRR //	EEIVHMGNAIMSFYS //	RASASLDTASLSVTE
RyR1 human	RSEGEKVRVGDDLIL //	LYNQFIKSLDSESGKPR //	ENRVHLGHAIMSFYA //	RAAASLDTATFSTTE
RyR1 pig	RSEGEKVRVGDDLIL //	LYNHFIKGLDSESGKPR //	ENRVHLGHAIMSFYA //	RAAASLDTATFSTTE
RyR1 rabbit	RSEGEKVRVGDDLIL //	LFNQFIKGLDLSGKKNK //	ENRVHLGHAIMSFYA //	RAAASLDTATFSTTE
RyR1 fish	RSEGEKVRVGDDLIL //	LFNQFIKGLDLSGKKNK //	ENRVHLGNAIMSFYS //	RAAASLDTVAEFTTE
RyR3 human	RSEGEKVRIGDDLIL //	LFSQFVSG-----NNR //	EEIVHMGNAIMSFYS //	RASASLDTVSLSTTE
RyR3 vison	RSEGEKVRIGDDLIL //	LFSQFVSG-----NNR //	EEIVHMGNAIMSFYS //	RASASLDTVSLSTTE
RyR3 rabbit	RSEGEKVRIGDDLIL //	LFSQFVSG-----NNR //	EEIVHMGNAIMSFYS //	RASASLDTVALSTTE
RyR3 chicken	RSEGEKVRIGDDLIL //	LFSQFISG-----NNR //	EEIVHMGNAIMSFYS //	RASASLDTVSLSTTE

**Figure 3.** Conservation of the RyR2 regions where ARVD2 mutations were detected. Amino acid sequence alignments of different RyRs from various species are shown for each protein segment where non-polymorphic RyR2 mutations occurred. Amino acids divergent from hRyR2 are shaded. The positions of Arg176, Leu433, Asn2386 and Thr2504 ARVD2 mutations are indicated by arrows. RyR2 sequences were reported so far only for humans and rabbits. According to protein pattern identification analysis, the Leu433Pro substitution introduces a destabilizing amino acid residue in a predicted  $\alpha$ -helix domain. Threonine 2504 is phosphorylated by CKI and GSK3 in all vertebrates, according to the consensus sequences Sp/Tp-X2-3-S/T-X and X-S/T-X-X-X-Sp, respectively. The substitution by a methionine in ARVD2 family 115 would introduce a defect in phosphorylation.



**Figure 4.** Schematic representation of the human RyR2 monomer and mutations identified in ARVD2 patients. The main features of the RyR2 protein are represented to scale. The predicted structure, very similar to that reported for RyR1, includes a cytoplasmic and a transmembrane region, separated by a calcium binding domain. Boxes represent the FKBP12.6 and calcium binding sites and the six transmembrane domains crossing the SR membrane. Arrows indicate the position of the four mutations detected in the present study in ARVD2 patients, whereas stars show the position of four missense polymorphisms found in ARVD2 families and the control population. The figure also indicates the RyR2 portions corresponding to three RyR1 MH/CCD regions, where most reported RyR1 point mutations linked to MH and CCD were detected (12).

overload, which should mostly affect the right ventricle because of the different thickness of the free wall. Therefore, physical exercise, which implies both increase of the heart rate and right-ventricular volume overload, might represent an important trigger of degenerative pathways and of the peculiar electrical instability observed in ARVD2.

The possibility that halogenated anesthetics could trigger an adverse response in ARVD2 patients, as in MH/CCD carriers, cannot be ruled out at present. In a recent report on the retrospective analysis of 50 cases of sudden unexpected perioperative deaths, arrhythmogenic right ventricular cardiomyopathy was detected in 18 subjects (18), but it was impossible to establish how many of them were ARVD2.

A mutation in plakoglobin (19) was detected in Naxos disease (OMIM 601214), an autosomal recessive syndrome characterized by palmoplantar keratoderma, woolly hairs and an arrhythmogenic cardiomyopathy mimicking ARVD. The authors suggested that ARVDs could arise from the disruption

of the functional integrity of the cell adhesion complex. According to present data, this hypothesis seems not to hold true, at least for ARVD2.

The detection of RyR2 mutations causing ARVD2, reported in this paper, opens the way to pre-symptomatic detection of carriers of the disease. In families in which a single ARVD2 case was diagnosed, DNA tests could reveal which infants are carriers, thus enabling early monitoring and treatment. Moreover, the discovery that RyR2 is involved in ARVD2 might possibly lead to a specific and effective pharmacological treatment.

## MATERIALS AND METHODS

### Patients

Families with recurrence of ARVD2 were identified at the Department of Cardiology of the University of Padova (Italy). Diagnostic criteria were according to McKenna *et al.* (20). The

following non-invasive investigations were also performed on apparently healthy relatives: clinical examination, chest X-ray, 12-lead electrocardiograph (ECG), 24 h ambulatory ECG, signal-averaged ECG (SAECG), stress test and two-dimensional and Doppler echocardiography.

Subjects were classified as affected or unaffected. Those showing minor clinical findings, insufficient for diagnosis, were classified as uncertain.

### Linkage studies

Genomic DNA samples were amplified by PCR using markers obtained from the Généthon and Genome Data Base (GDB) list of microsatellites or commercially available from Isogen Bioscience and Perkin-Elmer ABI PRISM, followed by electrophoresis on an ABI 373 automatic sequencer with automatic allele sizing using the GeneScan software (Applied Biosystems). Two-point linkage analyses were performed by the program MLINK of the LINKAGE software (version 5.2). For the multipoint analyses, the programs FASTLINK and FASTMAP were used. Information on the location of the different markers and genes on chromosome 1 map was obtained from the OMIM, GDB, Location Database (LDB) and WI/MIT databases (<http://www.ncbi.nlm.nih.gov/omim/> ; <http://www.gdb.org> ; [http://cedar.genetics.soton.ac.uk/public\\_html/ldb.html](http://cedar.genetics.soton.ac.uk/public_html/ldb.html) ; <http://www-genome.wi.mit.edu> ). For linkage calculations, the penetrance value was set to 95%.

### Physical mapping of the 1q42–q43 region

CEPH yeast artificial chromosome (YAC) clones belonging to the 1q42–q43 region, selected according to the WI/MIT database information about their location, were kindly provided by the DIBIT Centre (Milan, Italy). The YAC contig was assembled by STS content, using 16 markers (primer sequences available at Généthon and GDB databases). CEPH and ICI YAC clones close to the central gap of the contig and positive to *ACTN2* and *RYR2* genes were selected by PCR-based library screening. Reciprocal position of markers and candidate genes was established by integrating linkage data, radiation hybrid mapping (Genebridge4 Panel; Research Genetics) and PAC library screening (RPCI PAC library; UK-HGMP Centre, <http://www-hgmp.mrc.ac.uk> ).

### Bio-informatic analysis on candidate genes and primer design

The genomic structures of *ACTN2*, *NID* and *RYR2* were determined and primers designed for amplification from genomic DNA for mutation screening. The set of intronic primer pairs for mutation screening of *ACTN2* (GenBank accession no. AJ249756) had been described previously. The genomic structures of *NID* and *RYR2* genes were determined by direct comparison of their full-length cDNAs with the sequence of human chromosome 1 genomic clones, retrieved from the GenBank/htgs database (<http://www.ncbi.nlm.nih.gov> ) by BlastN search. *NID*-specific primer pairs for the 20 exons of the gene were designed for the cDNA sequence (GenBank accession no. M30269) and for the genomic clone RP4-746D2 (GenBank accession no. AL122018). *RYR2*-specific oligonucleotides for the 105 exons were designed by comparing the cDNA sequence (GenBank

accession no. NM\_001035) to nine overlapping genomic clones, sequenced at the WI/MIT Institute and at the Sanger Centre (GenBank accession nos AC074231, AC007990, AL445473, AC022248, AL354663, AC069110, AL365332, AL359924 and AL358212) (<http://www-genome.wi.mit.edu> ; <http://www.sanger.ac.uk> ).

PCR primers were designed by the OLIGO and PRIMER3 programs (<http://www-genome.wi.mit.edu> ). All the primer sequences are available at [http://telethon.bio.unipd.it/ARVDnet/molgen\\_arvd2.html](http://telethon.bio.unipd.it/ARVDnet/molgen_arvd2.html) ). The genomic structure of the *RYR2* gene is available from GenBank (accession nos AJ300340–AJ300444). The possible effect of amino acid changes on the structure of the RyR2 protein was assessed by BlastP comparison of RyR2 with other human, vertebrate and invertebrate RyR sequences. Protein pattern identification was performed by PIX analysis (<http://www.hgmp.mrc.ac.uk> ) and Phosphobase search.

### Mutation screening

Mutation screening was performed by direct sequencing, SSCP and dHPLC. Each exon was amplified from patient genomic DNA, purified (PCR Product Pre-Sequencing kit; USB) and sequenced using the BIG DYE dideoxy-terminator chemistry (Perkin Elmer) on an ABI 377 DNA sequencer (PE Applied Biosystems). Chromas 1.5 software (Technelysium) and LASERGENE package computer programs (DNASTAR) were used to edit, assemble and translate sequences. For each PCR fragment, dHPLC analysis was performed on a Transgenomic Wave DNA Fragment Analysis System using a DNASep column (Transgenomic). Column temperatures were calculated based on sequence and length in base pairs of the product using the WAVEmaker software. The separation conditions for analysis were a flow rate of 0.9 ml/min and a gradient (%B per min) adjusted to elute the amplicon between 3.5 and 6 min (see <http://stephan.childrens-research.org> for dHPLC programs for each amplicon). Samples were assumed to be heteroduplexes due to the dominant nature of the disease and unaffected DNA was not spiked into each reaction. SSCP analysis for *in cis* point mutations was performed under the following conditions: 5 µl of a 35 cycle PCR mixture was denatured and separated on a non-denaturing 10% polyacrylamide gel (29:1 acrylamide:bisacrylamide, 5% glycerol), in 1× TBE buffer at room temperature. Single strand conformations were detected by silver staining. Intragenic deletions were tested for by amplifying single exons of each candidate gene by multiplex semi-quantitative PCR: 100 ng of genomic DNA were used as template in 12.5 µl of 1× PCR buffer (16.6 mM ammonium sulfate, 67 mM Tris–HCl pH 8.3, 0.01% Tween-20, 1.5 mM magnesium chloride), containing a set of three primer pairs (each oligonucleotide at 800 nM), dNTPs at 100 µM and 0.5 U of *ExperTaq* polymerase (Experteam). Cycling conditions (denaturation at 94°C for 1 min, annealing at the working temperature for 1 min, extension at 72°C for 1 min) were repeated 18–22 times. After PCR, 12 µl of reaction mixture were separated on a 8% non-denaturing polyacrylamide gel. The gels were then silver-stained and dried.

### Population screening

In order to verify the occurrence of the detected mutations as normal variants within the Italian population, SSCP analysis

was performed for each mutation or, alternatively, a series of allele-specific primers was designed and used for PCR amplification of DNAs from affected cases belonging to the four ARVD2 families subjected to the study and from 120 genomic DNAs from unrelated healthy controls from the Venetian population. The same samples were PCR amplified using normal primers specific for the wild-type exon under investigation. PCR products were separated on a 2% horizontal agarose gel in 1× TAE buffer. In parallel, mutation screening was performed by dHPLC, as described above, on a series of 96 DNA specimens obtained from Italian Olympic athletes from various parts of Italy, known to not have cardiac dysfunction.

## ACKNOWLEDGEMENTS

We thank G.P. Marcon, R. Zimbello and I. Makalowska for technical assistance and all the ARVD2 families for their enthusiastic participation in the study. The financial support of Téléthon-Italy to A.N. (Project E-743), of MURST (Project: Molecular Genetics of Arrhythmic Cardiomyopathies) to G.A.D. and of the Fondazione Cassa di Risparmio di Padova e Rovigo is gratefully acknowledged. G.L. was supported by a SOCRATES student fellowship. A.R., N.T. and M.M. were supported by a post-doctorate salary by the University of Padua. B. Bauce is a PhD student at the University of Padua. American Heart Association support (0030118N) to D.A.S. and K.B. is acknowledged. B. Brahmhatt was supported by a fellowship from the Society of Experimental Biology and Medicine.

## NOTE ADDED IN PROOF

The recent finding of RYR2 mutations in patients showing catecholaminergic polymorphic ventricular tachycardia [S. Priori *et al.* (2000) *Circulation*, **102**, r49–r53] suggests the allelism of ARVD2 to this disease.

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