Journal of the American College of Cardiology Vol. 54, No. 10, 2009

Published by Elsevier Inc.
ISSN 0735-1097/09/$36.00
doi:10.1016/j.jacc.2009.05.038

Mutations in Ribonucleic Acid Binding Protein Gene Cause Familial Dilated Cardiomyopathy

Katharine M. Brauch, MS,* Margaret L. Karst, BA,* Kathleen J. Herron, BA,* Mariza de Andrade, PhD,§ Patricia A. Pellikka, MD,† Richard J. Rodeheffer, MD,† Virginia V. Michels, MD,‖ Timothy M. Olson, MD*†‡

Rochester, Minnesota

Objectives We sought to identify a novel gene for dilated cardiomyopathy (DCM).

Background DCM is a heritable, genetically heterogeneous disorder that remains idiopathic in the majority of patients. Familial cases provide an opportunity to discover unsuspected molecular bases of DCM, enabling pre-clinical risk detection.

Methods Two large families with autosomal-dominant DCM were studied. Genome-wide linkage analysis was used to identify a disease locus, followed by fine mapping and positional candidate gene sequencing. Mutation scanning was then performed in 278 unrelated subjects with idiopathic DCM, prospectively identified at the Mayo Clinic.

Results Overlapping loci for DCM were independently mapped to chromosome 10q25-q26. Deoxyribonucleic acid sequencing of affected individuals in each family revealed distinct heterozygous missense mutations in exon 9 of *RBM20*, encoding ribonucleic acid (RNA) binding motif protein 20. Comprehensive coding sequence analyses identified missense mutations clustered within this same exon in 6 additional DCM families. Mutations segregated with DCM (peak composite logarithm of the odds score >11.49), were absent in 480 control samples, and altered residues within a highly conserved arginine/serine (RS)-rich region. Expression of RBM20 messenger RNA was confirmed in human heart tissue.

Conclusions Our findings establish *RBM20* as a DCM gene and reveal a mutation hotspot in the RS domain. *RBM20* is preferentially expressed in the heart and encodes motifs prototypical of spliceosome proteins that regulate alternative pre-messenger RNA splicing, thus implicating a functionally distinct gene in human cardiomyopathy. *RBM20* mutations are associated with young age at diagnosis, end-stage heart failure, and high mortality. (J Am Coll Cardiol 2009;54:930–41) © 2009 by the American College of Cardiology Foundation

Prevention of heart failure has been a major public health focus, founded on knowledge of pathogenic mechanisms and modifiable risk factors for hypertension and coronary artery disease (1). Heart failure remains an idiopathic condition, however, in 50% of adults (2) and 66% of children (3) referred to cardiologists, and end-stage idiopathic dilated cardiomyopathy (DCM) is the most common indication for cardiac transplantation (4,5). Indeed, onset of heart failure symptoms in DCM typically portends advanced myocardial disease and risk for sudden death (6) after years to decades of clinically silent but insidiously progressive myopathy. Even in children, this inherent delay in diagnosis and treatment of DCM accounts for 10-year transplantation-free survival of only 42% (3). Improved prediction, treatment, and prevention of DCM will require discovery of pre-clinical biomarkers, better tools for risk-stratification, and the molecular and cellular basis of disease to enable mechanism-based therapies (1).

See page 942

Recognition of DCM as a familial disorder in 20% to 48% of cases (7–11) has provided a rationale for routine screening echocardiography in at-risk relatives to detect pre-symptomatic disease (12). Moreover, it has been the impetus for human genetics investigations to uncover the molecular basis of DCM (13,14). Since 1993, pathogenic mutations in over 20 genes encoding cytoskeletal, contractile, nuclear membrane, calcium-regulating, and ion channel proteins have been identified in patients with DCM (15). The majority of studies are hypothesis-based, targeting

From the *Cardiovascular Genetics Laboratory, †Division of Cardiovascular Diseases, Department of Internal Medicine, ‡Division of Pediatric Cardiology, Department of Pediatric and Adolescent Medicine, §Department of Health Sciences Research, and the ‖Department of Medical Genetics, College of Medicine, Mayo Clinic, Rochester, Minnesota. This work was supported by the National Heart, Lung, and Blood Institute, National Institutes of Health (R01 HL071225), and the Marriott Program for Heart Disease Research.

Manuscript received February 18, 2009; revised manuscript received April 7, 2009, accepted May 10, 2009.

candidate genes like *cardiac actin* (16) that encode proteins with established function in the heart. By contrast, unanticipated DCM genes and insights into disease pathobiology have emerged from rare families suitable for whole genome mapping studies (17–20). Here, we used genetic linkage analysis in 2 large families with autosomal-dominant DCM to map a disease locus, leading to discovery of a mutation hotspot within a ribonucleic acid (RNA)-binding protein gene associated with high morbidity and mortality.

Methods

Study subjects. Patients with DCM evaluated at the Mayo Clinic in the years 1987 to 1992 and 1999 to 2008 and their relatives were recruited, and medical records were reviewed. We enrolled 280 unrelated probands; familial DCM was confirmed in 24% (DCM documented in ≥1 first degree relative) and suspected in 27% (on the basis of history alone). Family history of sudden death was present in 18%. The 8 families described in the current study were white and of northern European ancestry by self-reporting. An ethnically matched group of 480 control subjects with normal echocardiograms was randomly selected from a community-based cohort (21). Subjects provided written informed consent under research protocols approved by the Mayo Clinic Institutional Review Board.

Echocardiograms in relatives were performed for clinical indications or under the auspices of the research study. Diagnostic criteria for DCM were: lack of an identifiable cause for disease, left ventricular diastolic and/or systolic dimensions >95th percentile indexed for body surface area (22), and left ventricular ejection fraction <50%. Subjects with normal echocardiograms were classified as "unaffected," and those with equivocal or insufficient data were classified as "uncertain." Genomic deoxyribonucleic acid (DNA) was isolated from peripheral-blood white cells (Puregene Blood Kit, Gentra/QIAGEN, Valencia, California) or from paraffin-embedded tissue (QIAamp DNA FFPE Tissue Kit, QIAGEN).

Linkage analysis and fine mapping. Genome-wide linkage analysis was performed with the ABI PRISM Linkage Mapping Set MD10, version 2.5 (Applied Biosystems, Foster City, California), consisting of polymerase chain reaction (PCR) primer pairs for 400 short tandem repeat markers. After PCR amplification of DNA samples, fragments were resolved on an ABI PRISM 3130xl, and genotypes were scored with GeneMapper Software (Applied Biosystems). Two-point and multipoint linkage analyses were performed using the FASTLINK program and specification of the following variables: a phenocopy rate of 0.001, equal marker allele frequencies, and dichotomous liability classes ("affected" and "unaffected"). For mutations, a frequency of 0.001 was specified. Logarithm of the odds (LOD) scores were determined for affected subjects only and for 80% and 100% penetrance models at recombination frequencies of 0.0 to 0.4.

Fine locus mapping was performed with microsatellite markers on physical maps, accessible on the website of the National Center for Biotechnology Information (NCBI) (23). Genotyping was accomplished by PCR amplification of DNA radiolabeled with [alpha^{32}P] deoxycytidine triphosphate, resolution of alleles by polyacrylamide-gel electrophoresis, and visualization by autoradiography. Scored genotypes were assembled as haplotypes to define the critical region.

Mutation detection and haplotype analysis. Expression profiles of candidate genes, derived from Affymetrix GeneChip array data for 12 normal human tissues (accession GDS424) or 61 normal mouse tissues (accession GDS592), were assessed by searching the Gene Expression Omnibus (GEO) link on the NCBI website (24). The genomic structure of *RBM20* was based on predicted reference messenger ribonucleic acid (mRNA) sequence (accession NM_001134363.1), retrieved from NCBI. Primer pairs were designed for genomic DNA PCR-amplification of the coding regions of the 14 predicted exons (Online Table 1), with Oligo Primer Analysis Software, version 6.71 (Molecular Biology Insights, Cascade, Colorado). For sequencing, amplified products were treated with ExoSAP-IT (USB Corp., Cleveland, Ohio) and sequenced by the dye-terminator method with use of an ABI PRISM 3730xl DNA Analyzer (Applied Biosystems). The DNA sequences were viewed and analyzed with Sequencher, version 4.5 DNA analysis software (Gene Codes Corp., Ann Arbor, Michigan). The reference mRNA and derived protein sequence (accession NP_001127835.1) were used for annotation of identified mutations.

Denaturing high-performance liquid chromatography (DHPLC) heteroduplex analysis (WAVE DHPLC System, Transgenomic, Omaha, Nebraska) was used to screen for sequence variants in our DCM cohort and control samples. Ideal buffer gradients and column melting temperatures were determined with Transgenomic Navigator software version 1.7.0 Build 25 and subsequent optimization (Online Table 1). Chromatographic elution profiles of amplified fragments were compared against the wild-type homoduplex pattern; samples yielding anomalous traces were selected for sequencing. To test for a common founder among families with the same *RBM20* mutation, haplotypes for mutant alleles were constructed from an intragenic

Abbreviations and Acronyms

cDNA = complementary deoxyribonucleic acid
DCM = dilated cardiomyopathy
DHPLC = denaturing high-performance liquid chromatography
DNA = deoxyribonucleic acid
GEO = Gene Expression Omnibus
ICD = implantable cardioverter-defibrillator
LOD = logarithm of the odds
mRNA = messenger ribonucleic acid
NCBI = National Center for Biotechnology Information
PCR = polymerase chain reaction
RNA = ribonucleic acid
RS = arginine/serine

JACC Vol. 54, No. 10, 2009
September 1, 2009:930–41

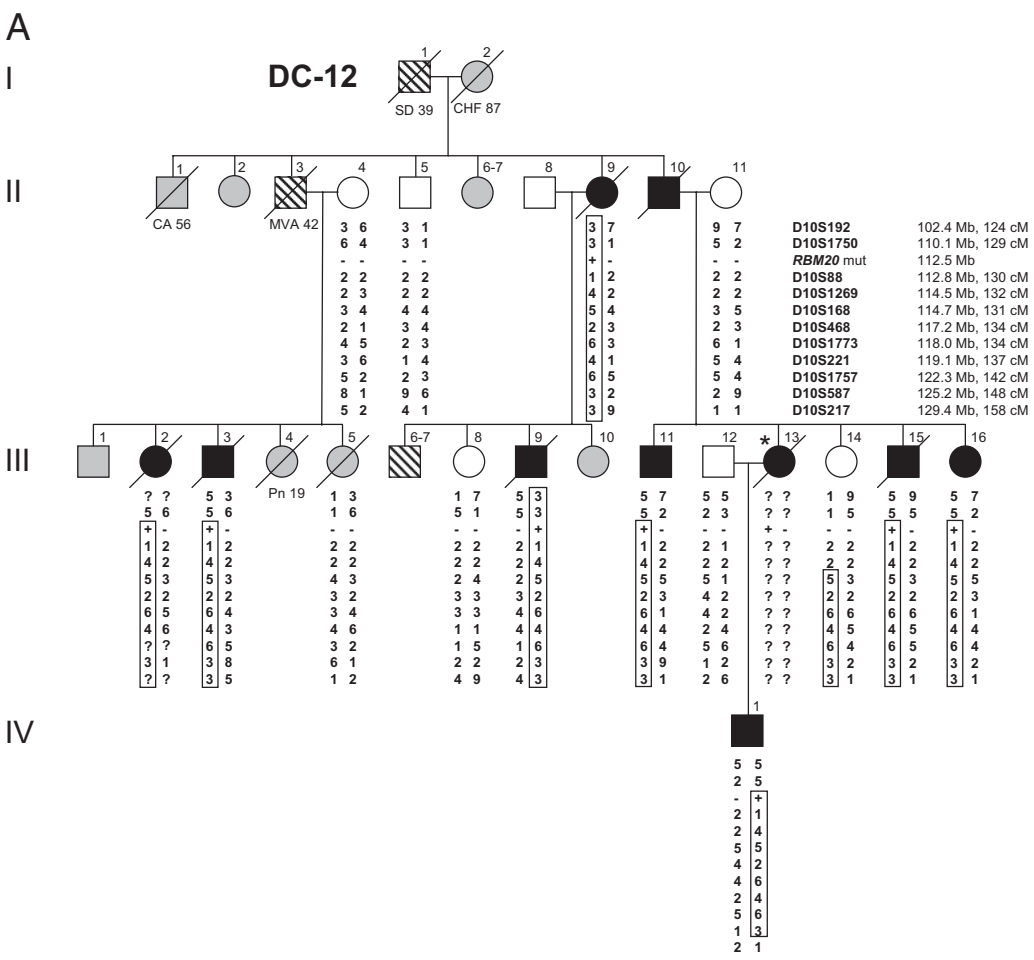

Figure 1 **Pedigrees of Index Families With Hereditary Dilated Cardiomyopathy**

Pedigree structures for kindreds DC-12 **(A)** and DC-35 **(B)** are shown. **Square** = male; **circle** = female; **solid** = affected; **open** = unaffected; **gray** = clinical status unknown; **parallel diagonal lines** = suspected dilated cardiomyopathy (DCM) on the basis of family history; **slash through the symbol** = deceased, with cause of/age at death indicated. The gene for ribonucleic acid binding motif protein 20 (*RBM20*) is located at chromosome 10q25.2. Markers that were tested for this region of chromosome 10 are listed in order from centromere to q-telomere, with map locations according to the National Center for Biotechnology Information website and given in megabases and centimorgans. The haplotypes for these markers are shown in **columns** beneath family members who underwent genetic evaluation; the disease-associated haplotypes are **boxed**. **Question marks** indicate genotypes that could not be scored from paraffin-embedded samples. Recombination events account for inheritance of portions of the disease haplotype in some affected subjects (DC-12: III.2, III.3, III.11, III.15, III.16, IV.1; DC-35: III.6, IV.1, IV.5, IV.9, IV.10) and enable critical regions to be defined by the minimal region of overlap. The *RBM20* missense mutations (*RBM20* mut), which cosegregate with DCM, are indicated by **plus symbols**; **minus symbols** indicate wild-type sequence. *Proband. ALZ = Alzheimer's disease; CA = cancer; CHF = congestive heart failure; CVA = cerebrovascular accident; MI = myocardial infarction; MS = multiple sclerosis; MVA = motor vehicle accident; Pn = pneumonia; SD = sudden death; Tx = cardiac transplantation.

Continued on next page.

tetranucleotide-repeat sequence and single nucleotide polymorphisms, identified by sequencing family members.

Cardiac mRNA expression and protein structure analysis. Total RNA was extracted from frozen human heart tissue (RNeasy Fibrous Tissue Midi Kit, QIAGEN), and 1.0 μg was reverse transcribed with an oligo(dT) primer to produce complementary deoxyribonucleic acid (cDNA) from mRNA (SMART RACE cDNA Amplification Kit, Clontech, Mountain View, California). Primers cDNA-F (CCTACCCCAGATCATCCAAAATGC) and cDNA-R (AACAAACACTTTGCAGTCAGTTATACA) were designed to PCR amplify and sequence 5′-RACE-Ready cDNA, spanning the *RBM20* region containing the identified mutations. A subsequent nested reaction with primers cDNA-2F (GAACCCATTCTCGGTCAGTAACCC) and cDNA-2F/3′UTR-R (TCTCTCTGCCCTTCCTCCATTAGT) was performed to provide optimal sequence quality. The RBM20 reference protein sequence was subjected to a Conserved Domain Database search performed with BLASTP, accessed on the NCBI website, to identify conserved structural domains. Conservation of amino acids altered by *RBM20* missense mutations was investigated by aligning our translated *RBM20* cDNA sequence with RBM20 protein sequences of other species.

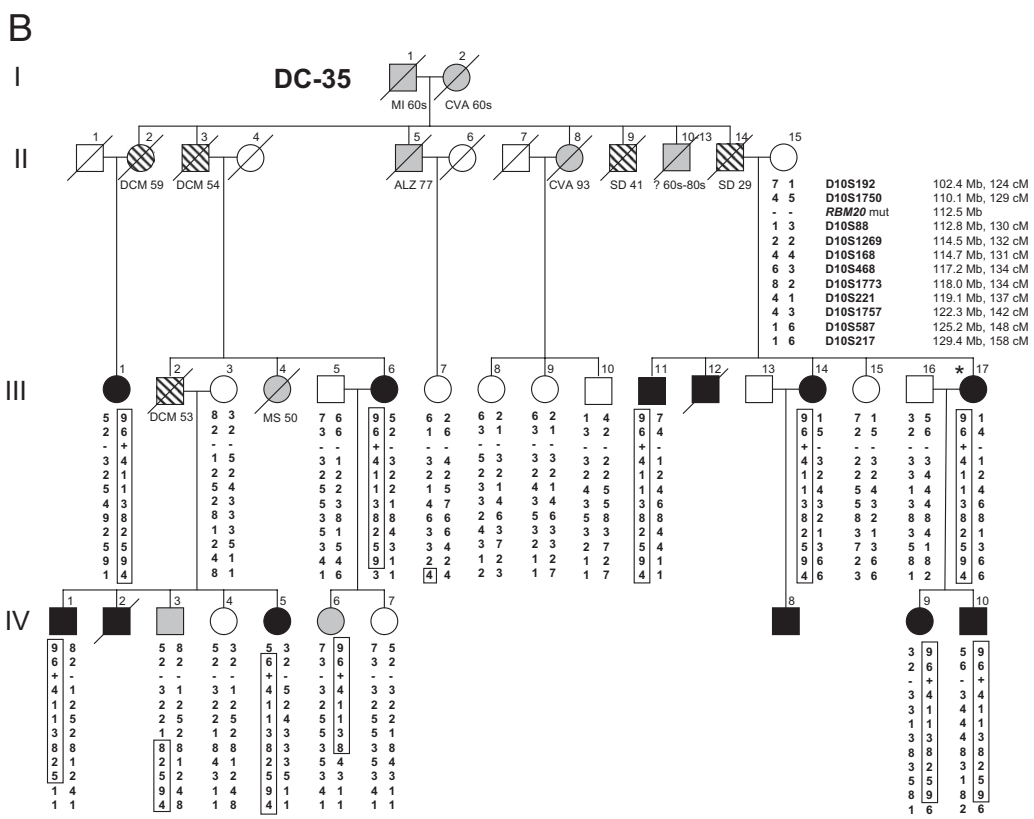

Figure 1 Continued

Results

Phenotype of index families. Clinical data and DNA samples were collected from 2 large families in which a clinically aggressive form of DCM segregated as an autosomal-dominant trait (Fig. 1, Table 1). Kindred DC-12 was recruited for the study in 1991, when an unaffected family member sought medical genetics consultation. The patriarch (Fig. 1A: I.1) was of Scottish ancestry and died suddenly at age 39 years. Ten family members developed documented DCM, 2 as young children (mean age at diagnosis: 30.0 years). Two underwent cardiac transplantation as young adults, and all but 3 have died of their disease (mean age at death: 37.7 years). Kindred DC-35 was recruited in 2005, after a diagnostic screening echocardiogram in the proband (Fig. 1B) (III.17) whose father died suddenly at age 29 years. The family was of Norwegian ancestry and comprised 12 relatives with documented DCM (mean age at diagnosis: 41.3 years) and 5 others with DCM and/or sudden death by history alone. Seven family members with confirmed or suspected DCM died at a mean age of 45.7 years. Five living relatives with DCM had received implantable cardioverter-defibrillators (ICDs).

DCM locus mapping. Genome-wide linkage analyses, followed by regional high-density genotyping on chromosome 10, identified a peak 2-point LOD score of 3.55 at marker *D10S1269* in DC-12 and 4.55 at marker *D10S221* in DC-35. Linkage to other regions of the genome with 2-point LOD scores >1.0 was excluded by multipoint and/or haplotype analyses with additional markers (data not shown). Fine mapping in DC-12 identified a disease-associated haplotype on chromosome 10q25.1-q26.2 (Fig. 1A), a region spanning 19.3 Mb, which was inherited by all affected subjects (peak multipoint LOD score 3.62 for all subjects, assuming 100% mutation penetrance, and 2.67 for affected subjects only). A recombination event within this interval occurred in a 43-year-old woman with a normal echocardiogram (III.14). The critical region narrowed to 4.6 Mb, assuming she did not inherit the disease-associated mutation. Fine mapping in DC-35 identified an overlapping disease-associated haplotype (Fig. 1B) spanning 22.8 Mb (peak multipoint LOD score 4.89 for all subjects, assuming 100% mutation penetrance, and 3.58 for affected subjects only). The haplotypes were different for each family, suggesting they did not share common ancestry, yet the overlapping disease loci raised the possibility of a shared DCM gene.

Mutation identification. Candidate genes were selected from the 19.3-Mb critical region in DC-12, comprising more than 150 genes, on the basis of cardiac expression and/or physiologic rationale. Mutations within exons of 25 genes were excluded by DNA sequencing (Online Table 2). *RBM20*, a gene with unknown function, was included on

Table 1 Phenotypic and Genetic Data for Families With DCM

Pedigree (Country of Origin)	Age at Diagnosis (yrs)	Age at Evaluation (yrs) (Indication)	LVID (mm)	LVEF (%)	ECG, Arrhythmia	Other Diagnostic Testing	Treatment	Outcome	Pathology	Diagnosis	RBM20 Mutation Status
DC-12 (Scotland)											
II.5	—	58 (F)	55/31	68	Normal		None	Alive 58 yrs		Unaffected	Normal
II.9	53	53 (R)	64*/53*	39	LVH, PVC			Death 58 yrs		DCM	P638L
II.10	44	45 (S)	Severe LVE*	LVSD	AF, PVC		D, F	CHF, death 45 yrs	Autopsy: congestive myopathy, fibrosis, myocyte hypertrophy, no CAD	DCM	P638L (inferred)
III.2	28	28 (S)						SD 28 yrs	Autopsy: EFE, congestive myopathy, no CAD	DCM	P638L
III.3	37	37 (F)	62*/57*	15	LAD, VT	Neg. angio	D, B	Death 41 yrs	Autopsy: mild fibrosis	DCM	P638L
III.5		30 (R)	40/24	64	Normal			Death 38 yrs	Autopsy: normal LV and cardiac mass, no CAD	Uncertain (suspected arrhythmia)	Normal
III.8		36 (F)	43/30	51	Normal		None	Alive 39 yrs		Unaffected	Normal
III.9	30's	36 (S)			LBBB		Transplant 36 yrs	Death 36 yrs		DCM	P638L
III.11	33	33 (F)	72*/62*	26	LVH, IVCD		D, C	Alive 42 yrs		DCM	P638L
III.13	29	29 (S)						SD 29 yrs	Autopsy: CM, mild fibrosis, no CAD	DCM	P638L
III.14		43 (F)	51/36	50	Normal		None	Alive 46 yrs		Unaffected	Normal
III.15	24	25 (F)	88*/79*	15	LVH, IVCD		Transplant 26 yrs	Death 27 yrs		DCM	P638L
III.16	14	14 (F)→ 22	57*/41*→ 57*/41*	48→ 45	Short PR, SVT		D, So	Alive 24 yrs		DCM	P638L
IV.1	3	3 (F)→ 12	44*/30*→ 62*/40*	50→ 64	Short PR, LVH		D, L	Alive 12 yrs		DCM	P638L
DC-35 (Norway)											
III.1	55	55 (F)	47/39*	46	PAC	Neg. stress imaging		Alive 58 yrs		DCM	R634Q
III.6	45	45 (S)→ 55	70*/→ 60*/50*	10→ 30	LAE, IVCD, ST-T	Neg. angio	D, Cv, L, Sp, W, ICD (EF, FH)	Alive 55 yrs		DCM	R634Q
III.7		60 (F)	44/29	60	Normal		None	Alive 62 yrs		Unaffected	Normal
III.8		60 (F)	39/29	60			None	Alive 60 yrs		Unaffected	Normal
III.9		52 (F)	Normal	65	Normal		None	Alive 56 yrs		Unaffected	Normal
III.10		51 (HTN)	44/28	67			None	Alive 52 yrs		Unaffected	Normal
III.11	55	55 (S)	72*/63*	20	Short PR, IVCD, ST-T, VT		D, Cv, L, ICD (EF, FH, VT)	Alive 55 yrs		DCM	R634Q
III.12	47	47 (A)						Death 47 yrs	Autopsy: CM and LV dilation	DCM	

Continued on next page

Table 1 Continued

Pedigree (Country of Origin)	Age at Diagnosis (yrs)	Age at Evaluation (yrs) (Indication)	LVID (mm)	LVEF (%)	ECG, Arrhythmia	Other Diagnostic Testing	Treatment	Outcome	Pathology	Diagnosis	RBM20 Mutation Status
III.14	46	52 (F)	63*/51*	30			ICD (EF, FH)	Alive 52 yrs		DCM	R634Q
III.15		51 (F)	54/27	55			None	Alive 51 yrs		Unaffected	Normal
III.17	48	48 (F)	61*/44*	45	IVCD, ST-T, VT	Neg. angio	M, L, ICD (FH)	Alive 49 yrs		DCM	R634Q
IV.1	50	50 (S)	64*/55*	20	LAD, LAE, ST-T	Neg. angio	Cv, L	Alive 52 yrs		DCM	R634Q
IV.2	37	37 (S)	Severe LVE*	15	LAE, LAD	Neg. angio, CK 102 U/l	D, E, W	CHF, death 37 yrs	Biopsy: myocyte hypertrophy, mild fibrosis	DCM	
IV.3		44 (F)	56*/36	65	Normal		None	Alive 48 yrs		Uncertain	Normal
IV.4		44 (F)	51/32	52	Normal		None	Alive 46 yrs		Unaffected	Normal
IV.5	40	40 (F)	56*/43*	40	Normal	Neg. stress imaging; CK 70 U/l, cTnI <0.3 ng/ml	M, L	Alive 44 yrs		DCM	R634Q
IV.6		24 (F)	54*/35	58	Normal	Neg. stress imaging	None	Alive 27 yrs		Uncertain	R634Q
IV.7		23 (F)	39/27	65			None	Alive 23 yrs		Unaffected	Normal
IV.8	18	18 (F)	61*/51*	37	IVCD, LVH	Neg. angio	Cv, L, ICD (FH, EF)	Alive 19 yrs		DCM	
IV.9	30	30 (R)	63*/45*	42	Normal			Alive 30 yrs		DCM	R634Q
IV.10	24	24 (R)	59/46*	45	LVH			Alive 24 yrs		DCM	R634Q
DC-50 (Germany)											
II.3	49	52 (S)→ 60	68*/62*→ 71*/65*	17→ 15	LVH, ST-T, AF, VT, VF	Neg. angio, CK 43 U/l, cTnI <0.5 ng/ml	D, F, P, C, A, W, ICD (Sy, FH)	CHF, death 60 yrs	Autopsy: sev. CM, mild fibrosis	DCM	P638L
II.5	29	29 (S)			ST-T, VT, VF	CK 29 U/l	D, P, PC	CHF, death 29 yrs		DCM	P638L (inferred)
III.3	25	25 (R)→ 42	55*/45*→ 51/—	33→ 40	LVH		D, Cv, L	Alive 42 yrs		DCM	P638L
III.4	29	29 (R)→ 44	45/35→ 52/38*	40→ 49	ST-T		Cv, E	Alive 44 yrs		DCM	P638L
III.5	15	15 (S)	75*/68*	18	LVH, ST-T, VT		D, L, W, N, Mx	CHF, SD 18 yrs	Biopsy: myocyte hypertrophy, mild fibrosis	DCM	
III.6		29 (R)	51/28	70	Normal		None	Alive 46 yrs		Unaffected	Normal
III.7		27 (R)	50/32	60	Normal		None	Alive 36 yrs		Unaffected	Normal
III.8	21	27 (R)→ 37	54*/40*→ 56*/46*	51→ 35	Short PR, SVT		Cv, L	Alive 38 yrs		DCM	P638L
IV.1	17	17 (S)	51/40*	40	LVH, SVT		M, E	Alive 22 yrs		DCM	P638L
DC-46 (Germany)											
IV.1	26	18 (F)→ 26	53/36→ 56*/46*	58→ 30	ST-T	cTnT <0.03 ng/ml	Cv, L, ICD (EF, FH)	Alive 27 yrs		DCM	R636S

Continued on next page

Table 1 Continued

Pedigree (Country of Origin)	Age at Diagnosis (yrs)	Age at Evaluation (yrs) (Indication)	LVID (mm)	LVEF (%)	ECG, Arrhythmia	Other Diagnostic Testing	Treatment	Outcome	Pathology	Diagnosis	RBM20 Mutation Status
DC-49 (Germany)											
II.2	40	40 (S)→ 45	71*/64*→ 65*/52*	10→ 20	LVH, ST-T, IVCD, VT		D, M, L, Sp, F, W, Mx, ICD (CA)	Alive 45 yrs		DCM	R636H
II.3	39	39 (F)→ 44	63*/49*→ 52/42*	43→ 45	VT	Neg. angio	Cv, Rm, Cn	Alive 44 yrs		DCM	R636H
DC-27 (Norway)											
II.6	70	80 (S)	63*/—	25	SB, AF		D, Cv, L, F, W	CHF, death 85 yrs		DCM	R636S
III.2		64 (F)	55*/40*	50	IRBBB		Cv	Alive 64 yrs		Uncertain	R636S
III.3	59						None	SD 59 yrs	Autopsy: CM, LVE, CAD but no acute MI, fibrosis	DCM	R636S (inferred)
III.5	55	59 (F)	59*/44*	44	1° AVB, RBBB, VT	Neg. angio	Cv	Alive 60 yrs		DCM	R636S
III.8		50 (F)	45/—	60	Normal		None	Alive 55 yrs		Unaffected	Normal
III.10		39 (F)	54/38*	55			None	Alive 47 yrs		Uncertain	R636S
IV.1	35	35 (S)	68*/55*	38	LVH, ST-T	Neg. angio	Cv, L	Alive 36 yrs		DCM	R636S
IV.5	27	36 (S)	72*/65*	23	LAD, IVCD, ST-T	Neg. angio	Cv, Ln	Alive 37 yrs	Biopsy: myocyte hypertrophy, mod. fibrosis	DCM	R636S
IV.7		28 (R)	50/33	66	Normal		None	Alive 31 yrs		Unaffected	Normal
IV.9		15 (F)	52*/34	57	Normal		None	Alive 18 yrs		Uncertain	R636S
DC-09 (Norway)											
III.2	57	57 (R)→ 68	58*/46*→ 59*/49*	35→ 34	Short PR, PVC		D, E, F, A	Alive 68 yrs		DCM	R636S
III.4						Neg. angio		Alive 68 yrs		DCM (by history)	R636S
IV.2	17	17 (S)	68*/60*	22	LVH, ST-T		D, H, N, F, W	CHF, SD 18 yrs		DCM	
IV.3		27 (R)	50/32	60	Normal		None	Alive 38 yrs		Unaffected	R636S
IV.4		24 (R)	53/33	61	Normal		None	Alive 36 yrs		Unaffected	Normal
IV.6	19	20 (S)					Transplant 20 yrs	Alive 43 yrs		DCM	R636S
DC-22 (England)											
II.2	44	45 (S)	53*/44*	25	ST-T, VT	Neg. angio	D, F, A, Cv, Ln, ICD (EF, FH)	CHF, alive 54 yrs		DCM	S637G
II.3	27	27 (S)					Transplant 32 yrs	Alive 49 yrs		DCM	S637G

Continued on next page

Table 1 Continued

Pedigree (Country of Origin)	Age at Diagnosis (yrs)	Age at Evaluation (yrs) (Indication)	LVID (mm)	LVEF (%)	ECG, Arrhythmia	Other Diagnostic Testing	Treatment	Outcome	Pathology	Diagnosis	RBM20 Mutation Status
III.1	21	21 (F)	53*/39*	35	ST-T		Cv, L	Alive 28 yrs		DCM	S637G
III.2	20	20 (F)	48/34*	46	Short PR	Neg. stress imaging	Cv	Alive 23 yrs		DCM	S637G

*Left ventricular internal dimension measurement in diastole/systole >95th percentile, on the basis of body surface area and age.

Indication for evaluation: A = autopsy; F = family history; HTN = hypertension; S = symptoms; R = research study. Echocardiography: LVE = left ventricular enlargement; LVEF = left ventricular ejection fraction (normal ≥50%); LVID = left ventricular internal dimension in diastole/systole; LVSD = left ventricular systolic dysfunction. Electrocardiogram, arrhythmia: 1° AVB = first degree atrioventricular block; AF = atrial fibrillation; IRBBB = incomplete right bundle branch block; IVCD = intraventricular conduction delay; LAD = left axis deviation; LAE = left atrial enlargement; LBBB = left bundle branch block; LVH = left ventricular hypertrophy; PAC = premature atrial contractions; PR = PR interval; PVC = premature ventricular contractions; SB = sinus bradycardia; ST-T = nonspecific ST-T wave changes; SVT = supraventricular tachycardia; VF = ventricular fibrillation; VT = ventricular tachycardia. Other diagnostic testing: CK = creatine kinase; cTnI = cardiac troponin I; cTnT = cardiac troponin T; Neg. angio = no significant coronary artery disease on angiography. Treatment: A = amiodarone; B = benazepril; C = captopril; Cn = candesartan; Cv = carvedilol; D = digoxin; E = enalapril; F = furosemide; H = hydralazine; ICD = implantable cardioverter-defibrillator; L = lisinopril; Ln = losartan; M = metoprolol; Mx = mexiletine; N = nitroglycerin; P = propranolol; PC = procainamide; Rm = ramipril; So = sotalol; Sp = spironolactone; Transplant = cardiac transplantation; W = warfarin. Indication for ICD (in parentheses): CA = cardiac arrest; EF = ejection fraction; FH = family history; Sy = syncope; VT = ventricular tachycardia. Outcome: CHF = congestive heart failure; SD = sudden death. Pathology: CAD = coronary artery disease; CM = cardiomegaly; EFE = endocardial fibroelastosis; MI = myocardial infarction; mod. = moderate; Sev. = severe.

the basis of its genomic location and expression pattern. Among 12 human tissues, *RBM20* is most highly expressed in the heart, with transcript abundance 4-fold greater in cardiac than in skeletal muscle according to GEO array data. Moreover, it is 1 of only 19 genes with a mean expression in the heart >8-fold higher than the combined mean expression in 11 other tissues. Similarly, among 61 murine tissues it is most highly expressed in heart (>5-fold skeletal muscle). Sequencing of the 14 exons of *RBM20* identified a distinct heterozygous missense mutation in exon 9 in each family, resulting in a P638L substitution in DC-12 and a R634Q substitution in DC-35 (Figs. 1 and 3A). Mutations cosegregated with the disease phenotype and were absent in unaffected family members and 480 ethnically matched control subjects.

To determine whether *RBM20* mutations were present in other cases of DCM, we screened the 14 coding exons in our remaining cohort of 278 subjects with DHPLC. Three unique heterozygous missense mutations—R636S, R636H, and S637G—were identified in 6 other families, all clustered within exon 9 (Figs. 2 and 3A). Among the 8 families with *RBM20* mutations, 2 had an identical mutation resulting in P638L substitution, and 3 had an identical mutation resulting in R636S substitution. Haplotype analysis (Online Table 3) excluded a common ancestral founder for the P638L substitution. Although the disease-associated haplotypes were the same in the 3 families with an R636S substitution, the majority of individual alleles comprising the haplotype are the most common variants within a white European population. Consequently, a founder effect could not be conclusively established. Mutations were absent in control samples and cosegregated with DCM in the 7 families where DNA samples were available from 2 or more affected subjects. Combined peak 2-point LOD scores for mutations versus DCM in the 4 largest families (DC-12, DC-35, DC-27, DC-50) ranged from 8.02 (affected subjects only) to 11.49 (all subjects, assuming 100% mutation penetrance).

Cardiac mRNA expression and protein structure analysis. *RBM20* comprises, on the basis of the predicted reference cDNA (mRNA), 14 exons (Fig. 3B). Portions of exons 2 and 14 and all of exons 3 through 13 were verified in a single open reading frame cDNA derived from oligo(dT)-primed heart RNA (Fig. 3B). This confirmed that these exons are transcribed and spliced into mRNA in the heart, including exon 9, which contained the cluster of identified *RBM20* mutations. A Conserved Domain Database search of the translated reference *RBM20* cDNA indicated homology to an RNA Recognition Motif 1 Superfamily domain spanning exons 6 and 7 (e-value = 0.005) and a U1 zinc finger domain (e-value = $2e^{-4}$) spanning exons 13 and 14. Additionally, exon 9 encodes an arginine/serine (RS)-rich domain, which is disrupted by the 5 identified unique missense mutations. Each resultant amino acid substitution alters a residue in RBM20 conserved among diverse species (Fig. 3C).

JACC Vol. 54, No. 10, 2009
September 1, 2009:930–41

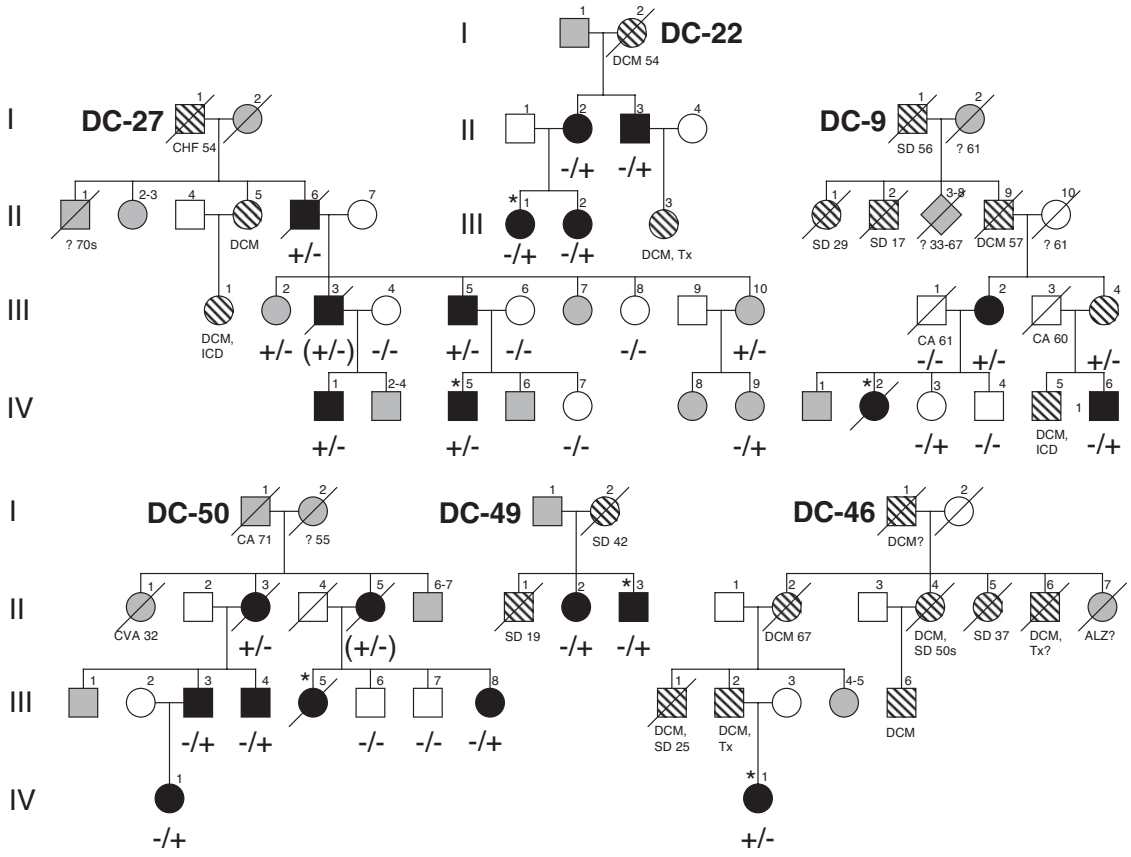

Figure 2 **Pedigrees of Additional Families With *RBM20* Mutations**

Diamonds = 2 or more family members of both sexes; **parentheses** = inferred *RBM20* mutation status. Symbols and abbreviations as in Figure 1.

Genotype-phenotype correlation. *RBM20* mutations were associated with clinically aggressive DCM. Collectively, the 39 subjects in our 8 families with a mutation and confirmed DCM were diagnosed 9 years earlier than a comparable series of patients with sporadic and familial DCM who underwent family screening (mean age at diagnosis 35.9 vs. 45.2 years) (7). Death occurred in 11 (mean age 45.2 years) and was deemed sudden in 3; 4 underwent cardiac transplantation (mean age 28.5 years); and 8 underwent ICD insertion. Subjects who enrolled in our study, however, did not fully represent the malignant nature of their familial disease as revealed by their pedigrees. Among the 32 additional relatives with suspected DCM by family history, for whom medical records were unavailable and/or mutation status could not be determined, 13 died suddenly (mean age 32.7 years), 3 underwent cardiac transplantation, and 3 had ICD insertion. There were no consistent electrocardiographic features in subjects with an *RBM20* mutation; 9 had ventricular tachycardia. Variable degrees of myocyte hypertrophy and interstitial fibrosis were observed on histopathological analysis. Most enrolled subjects with accessible follow-up data had advanced disease and exhibited minimal improvement or further deterioration on medical treatment, although drug therapy was highly variable. Correlation between *RBM20* mutations and phenotype was not without exception, however. There were 5 female subjects who inherited a mutation but did not fulfill diagnostic criteria for DCM: 1 subject in DC-35 (age 24 years) and 3 subjects in DC-27 (ages 15, 39, and 64 years) had left ventricular enlargement with normal ejection fraction; 1 subject in DC-9 (age 27 years) had a normal echocardiogram. No overt noncardiac phenotypes were evident among subjects with *RBM20* mutations.

Discussion

Molecular basis of disease. The majority of known DCM genes encode cytoskeletal or contractile proteins of cardiac myocytes, with direct roles in the generation and/or transmission of contractile force through protein–protein interactions (14). An expanded understanding of the pathobiology of DCM has emerged from identification of mutations that perturb myocardial function via impaired calcium (25), potassium (26), or sodium ion

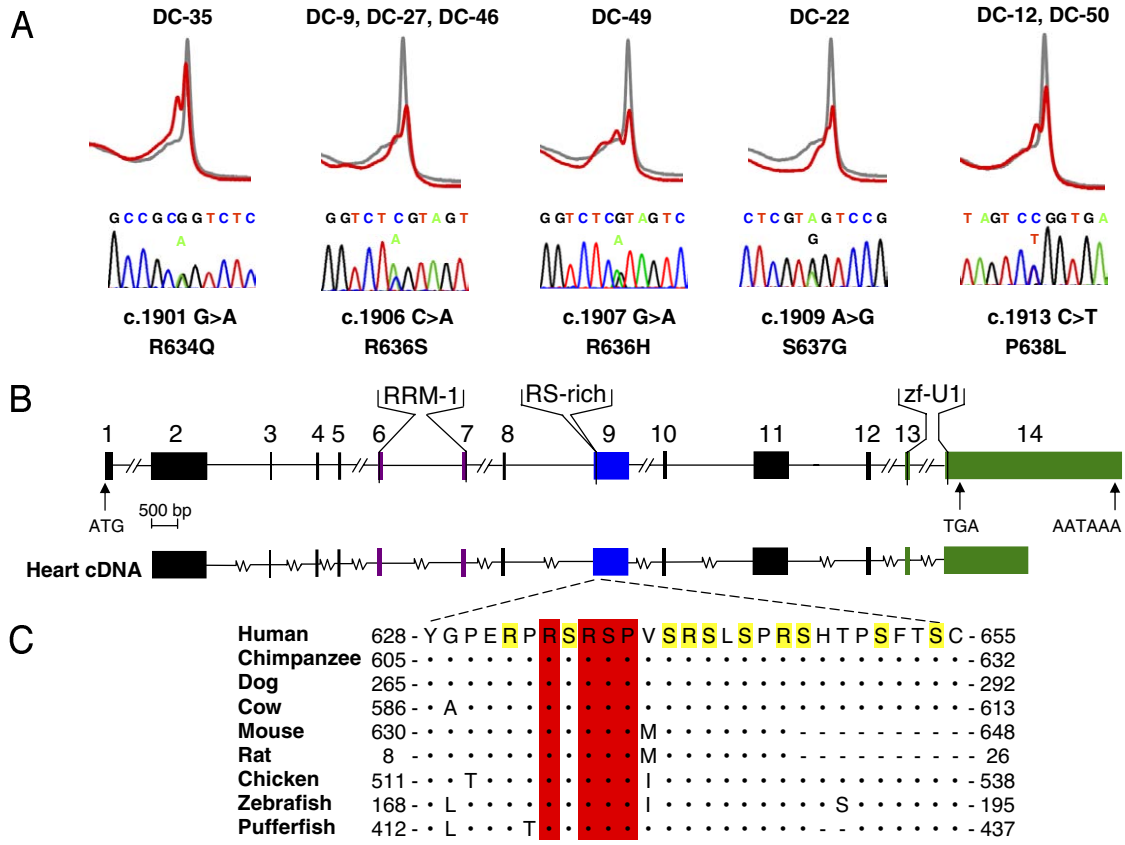

Figure 3 ***RBM20* Mutations Identified in Families With Dilated Cardiomyopathy**

(A) Genomic deoxyribonucleic acid (DNA) mutation scans with denaturing high-performance liquid chromatography revealed abnormal heteroduplex profiles **(red)**, compared with the control wild-type profile **(gray)**, in exon 9 of *RBM20* in 8 dilated cardiomyopathy families. Abnormal profiles indicated DNA sequence alterations, which were absent in 480 control samples. Below the heteroduplex profiles, DNA sequencing revealed corresponding heterozygous missense mutations. The location of each mutation and its resultant amino acid substitution are based on predicted reference *RBM20* complementary deoxyribonucleic acid (cDNA) and protein sequences and are indicated below each chromatogram. Mutation c.1906 C>A, R636S was shared by 3 families, and c.1913 C>T, P638L was shared by 2 families. **(B)** The predicted genomic structure of *RBM20*, consisting of 14 exons, is depicted to scale. Exons that encode peptides homologous to highly conserved functional domains—ribonucleic acid (RNA) recognition motif 1 (RRM-1, in **purple**), arginine/serine-rich region (RS-rich, in **blue**), and U1 zinc finger (zf-U1, in **green**)—are indicated. Putative start (ATG) and stop (TGA) codons are located in exons 1 and 14, respectively. A polyadenylation signal (AATAAA) is located at the 3' end of exon 14. Directly below the *RBM20* genomic structure, cDNA amplification and sequencing confirmed transcription of messenger RNA from exons 2 to 14 in human heart tissue, as depicted by parallel alignment. The cDNA transcript contains the complete RS domain and identified *RBM20* mutations in the 5' region of exon 9. **(C)** Alignment of homologous RBM20 protein sequences that flank the amino acid substitutions is shown. The RS domain spans residues 632 to 654, with arginine (R) and serine (S) residues highlighted in **yellow**. Residues conserved between human RBM20 and another species are indicated by **(•)** and amino acid deletions by **(−)**. Amino acids that are altered by the identified *RBM20* missense mutations (residues 634, 636, 637, and 638, indicated with **red bars**) are conserved among all 8 species. Accession numbers: NP_001127835.1 for human, XP_508032 for chimpanzee, XP_544017 for dog, XP_603772 for cow, BAE24961 for mouse, NP_001101081 for rat, XP_421755 for chicken, XP_683222 for zebrafish, and CAG01297 for pufferfish.

homeostasis (18,19). Collectively, these molecular genetic etiologies for DCM reveal a fundamental defect in excitation-contraction coupling and the heart's capacity to perform under physiologic and stress conditions. Notable exceptions to this paradigm have been revealed through discovery of unsuspected DCM genes, like *LMNA* and *EYA4*, in large families suitable for linkage analysis. *LMNA* encodes lamin A/C, a ubiquitously expressed nuclear membrane protein. By unknown mechanisms, mutations in *LMNA* cause DCM and conduction system disease (17) or a spectrum of noncardiac disorders. *EYA4* encodes a transcriptional coactivator, which interacts with DNA-binding transcription factors. Mutations in *EYA4* are predicted to alter cochlear and cardiac gene expression, causing a syndrome of DCM and sensorineural hearing loss (20). *RBM20*, here identified as a gene for familial DCM, suggests perturbation of post-transcriptional pre-mRNA processing as a distinct molecular basis for the disorder.

RBM20 encodes RNA binding motif protein 20, with a prototypical RNA-recognition motif followed by an RS domain (27). These structural features are characteristic of a family of RNA-binding SR proteins that assemble in the spliceosome, a large multiprotein complex that orchestrates constitutive and alternative splicing of pre-mRNA (28). Indeed, over 70% of human genes express

multiple mRNA transcripts via alternative splicing of exons, conferring vast diversity to the proteome (29). Heritable diseases are frequently attributable to *cis*-acting mutations, which disrupt normal splicing of the gene in which the mutation occurs. However, *trans*-acting mutations within spliceosome protein genes have been identified in only 3 human disorders—spinal muscular atrophy, retinitis pigmentosa, and Prader-Willi syndrome (28). Such mutations have the potential to impair normal splicing of multiple genes, as recently demonstrated by exon microarray analysis in a mouse model of spinal muscular atrophy (30). The specific function of RNA binding motif protein 20 in the human heart and the downstream effects of the identified *RBM20* mutations that cause DCM remain unknown. However, a pathogenic link between genetic disruption of alternative splicing-regulating SR proteins of the spliceosome and DCM has now been established in mouse models (31).

Clinical implications. Since the first DCM-associated gene was identified by linkage analysis over 15 years ago (32,33), clinical application of research findings has proved challenging due to the marked genetic heterogeneity of DCM. Although routine genetic testing might be practical in certain heritable cardiac disorders (34), no single gene or mutation for DCM has emerged as common (15). Targeted genetic testing might be practical, however, in clinically defined subgroups. For example, mutations in *LMNA* and *SCN5A* have been associated with a cardiac syndrome of DCM, impaired automaticity and conduction, and atrial fibrillation (17–19). By use of genome-wide linkage analysis, the present study further expands the spectrum of DCM genes. Remarkably, the 5 unique *RBM20* mutations identified in 8 families are clustered within a single exon that encodes an RS-rich domain. In our cohort, this mutation hotspot accounted for 3% (8 of 280) of all DCM cases, 5% (8 of 151) of confirmed or suspected familial cases, and 13% (7 of 54) of cases with a history of sudden death.

Our study highlights the importance of family screening to detect pre-symptomatic DCM (7,12). Indeed, 68% (43 of 63) of the subjects in our 8 families were asymptomatic and first diagnosed with DCM on the basis of a screening echocardiogram. Despite the lack of symptoms, the *RBM20* mutations we identified were highly penetrant, and only 5 of 44 individuals with a mutation did not fulfill diagnostic criteria for DCM. In fact, 4 of these 5 subjects had left ventricular dilation, a known precursor to overt DCM (7,10). However, penetrance of familial DCM is age dependent, and the majority of subjects who enrolled in our study were adults. Discovery of the genetic basis for DCM in these families now enables a pre-clinical diagnosis in at-risk children and young adults. Given the malignant nature of *RBM20* mutations, this knowledge would justify closer clinical follow up, meticulous attention to coexistent modifiable risk factors, and earlier institution of therapies proven to alter the natural history of heart failure (35) and decrease the risk of sudden death (6).

Acknowledgments

The authors gratefully acknowledge the patients and families who participated in this study and the physicians who referred them. The authors thank Jeanne L. Theis, PhD, for critical review of the paper.

Reprint requests and correspondence: Dr. Timothy M. Olson, Mayo Clinic, Stabile 5, 200 First Street Southwest, Rochester, Minnesota 55905. E-mail: olson.timothy@mayo.edu.

REFERENCES

1. Braunwald E. Cardiovascular medicine at the turn of the millennium: triumphs, concerns, and opportunities. N Engl J Med 1997;337:1360–9.
2. Felker GM, Thompson RE, Hare JM, et al. Underlying causes and long-term survival in patients with initially unexplained cardiomyopathy. N Engl J Med 2000;342:1077–84.
3. Towbin JA, Lowe AM, Colan SD, et al. Incidence, causes, and outcomes of dilated cardiomyopathy in children. JAMA 2006;296:1867–76.
4. Taylor DO, Edwards LB, Boucek MM, et al. Registry of the International Society for Heart and Lung Transplantation: twenty-fourth official adult heart transplant report—2007. J Heart Lung Transplant 2007;26:769–81.
5. Boucek MM, Aurora P, Edwards LB, et al. Registry of the International Society for Heart and Lung Transplantation: tenth official pediatric heart transplantation report—2007. J Heart Lung Transplant 2007;26:796–807.
6. Desai AS, Fang JC, Maisel WH, Baughman KL. Implantable defibrillators for the prevention of mortality in patients with nonischemic cardiomyopathy: a meta-analysis of randomized controlled trials. JAMA 2004;292:2874–9.
7. Michels VV, Moll PP, Miller FA, et al. The frequency of familial dilated cardiomyopathy in a series of patients with idiopathic dilated cardiomyopathy. N Engl J Med 1992;326:77–82.
8. Mestroni L, Krajinovic M, Severini GM, et al. Familial dilated cardiomyopathy. Br Heart J 1994;72:S35–41.
9. Keeling PJ, Gang Y, Smith G, et al. Familial dilated cardiomyopathy in the United Kingdom. Br Heart J 1995;73:417–21.
10. Baig MK, Goldman JH, Caforio AL, Coonar AS, Keeling PJ, McKenna WJ. Familial dilated cardiomyopathy: cardiac abnormalities are common in asymptomatic relatives and may represent early disease. J Am Coll Cardiol 1998;31:195–201.
11. Grünig E, Tasman JA, Kücherer H, Franz W, Kübler W, Katus HA. Frequency and phenotypes of familial dilated cardiomyopathy. J Am Coll Cardiol 1998;31:186–94.
12. Burkett EL, Hershberger RE. Clinical and genetic issues in familial dilated cardiomyopathy. J Am Coll Cardiol 2005;45:969–81.
13. Collins FS, McKusick VA. Implications of the human genome project for medical science. JAMA 2001;285:540–4.
14. Olson TM. Monogenic dilated cardiomyopathy. In: Walsh RA, editor. Molecular Mechanisms of Cardiac Hypertrophy and Failure. 1st edition. Boca Raton, FL: Taylor & Francis, 2005:525–40.
15. Hershberger RE, Parks SB, Kushner JD, et al. Coding sequence mutations identified in MYH7, TNNT2, SCN5A, CSRP3, LBD3, and TCAP from 313 patients with familial or idiopathic dilated cardiomyopathy. Clin Translational Science 2008;1:21–6.
16. Olson TM, Michels VV, Thibodeau SN, Tai YS, Keating MT. Actin mutations in dilated cardiomyopathy, a heritable form of heart failure. Science 1998;280:750–2.
17. Fatkin D, MacRae C, Sasaki T, et al. Missense mutations in the rod domain of the lamin A/C gene as causes of dilated cardiomyopathy and conduction-system disease. N Engl J Med 1999;341:1715–24.

18. McNair WP, Ku L, Taylor MRG, et al. *SCN5A* mutation associated with dilated cardiomyopathy, conduction disorder, and arrhythmia. Circulation 2004;110:2163–7.
19. Olson TM, Michels VV, Ballew JD, et al. Sodium channel mutations and susceptibility to heart failure and atrial fibrillation. JAMA 2005; 293:447–54.
20. Schönberger J, Wang L, Shin JT, et al. Mutation in the transcriptional coactivator EYA4 causes dilated cardiomyopathy and sensorineural hearing loss. Nat Genet 2005;37:418–22.
21. Redfield MM, Jacobsen SJ, Burnett JC, Mahoney DW, Bailey KR, Rodeheffer RJ. Burden of systolic and diastolic ventricular dysfunction in the community. Appreciating the scope of the heart failure epidemic. JAMA 2003;289:194–202.
22. Henry WL, Gardin JM, Ware JH. Echocardiographic measurements in normal subjects from infancy to old age. Circulation 1980;62:1054–61.
23. National Center for Biotechnology Information (NCBI). Available at: http://www.ncbi.nlm.nih.gov. Accessed February 20, 2008.
24. Barrett T, Troup DB, Wilhite SE, et al. NCBI GEO: archive for high-throughput functional genomic data. Nucleic Acids Res 2009; 37(Database issue):D885–90.
25. Schmitt JP, Kamisago M, Asahi M, et al. Dilated cardiomyopathy and heart failure caused by a mutation in phospholamban. Science 2003;299:1410–3.
26. Bienengraeber M, Olson TM, Selivanov VA, et al. ABCC9 mutations identified in human dilated cardiomyopathy disrupt catalytic KATP channel gating. Nat Genet 2004;36:382–7.
27. Long JC, Caceres JF. The SR protein family of splicing factors: master regulators of gene expression. Biochem J 2009;417:15–27.
28. Wang GS, Cooper TA. Splicing in disease: disruption of the splicing code and the decoding machinery. Nat Rev Genet 2007;8:749–61.
29. Johnson JM, Castle J, Garrett-Engele P, et al. Genome-wide survey of human alternative pre-mRNA splicing with exon junction microarrays. Science 2003;302:2141–4.
30. Zhang Z, Lotti F, Dittmar K, et al. SMN deficiency causes tissue-specific perturbations in the repertoire of snRNAs and widespread defects in splicing. Cell 2008;133:585–600.
31. Ding JH, Xu X, Yang D, et al. Dilated cardiomyopathy caused by tissue-specific ablation of SC35 in the heart. EMBO J 2004;23: 885–96.
32. Towbin JA, Hejtmancik JF, Brink P, et al. X-linked dilated cardiomyopathy. Molecular genetic evidence of linkage to the Duchenne muscular dystrophy (dystrophin) gene at the Xp21 locus. Circulation 1993;87:1854–65.
33. Muntoni F, Cau M, Ganau A, et al. Brief report: deletion of the dystrophin muscle-promoter region associated with X-linked dilated cardiomyopathy. N Engl J Med 1993;329:921–5.
34. Robin NH, Tabereaux PB, Benza R, Korf BR. Genetic testing in cardiovascular disease. J Am Coll Cardiol 2007;50:727–37.
35. Eichhorn EJ, Bristow MR. Medical therapy can improve the biological properties of the chronically failing heart. A new era in the treatment of heart failure. Circulation 1996;94:2285–96.

Key Words: dilated cardiomyopathy ■ genetics ■ linkage analysis ■ mutation ■ *RBM20*

APPENDIX

For supplementary Tables 1 to 3, please see the online version of this article.