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Research Letter**CRELD1 Mutations Contribute to the Occurrence of Cardiac Atrioventricular Septal Defects in Down Syndrome****Cheryl L. Maslen,^{1,2,3*} Darcie Babcock,¹ Susan W. Robinson,¹ Lora J. H. Bean,⁴ Kenneth J. Dooley,⁵ Virginia L. Willour,⁶ and Stephanie Sherman⁴**¹Department of Medicine, Division of Endocrinology, Oregon Health & Science University, Portland, Oregon²Department of Molecular and Medical Genetics, Oregon Health & Science University, Portland, Oregon³Heart Research Center, Oregon Health & Science University, Portland, Oregon⁴Department of Human Genetics, Emory University, Atlanta, Georgia⁵Department of Pediatrics, Sibley Heart Center, Cardiology, Children's Healthcare of Atlanta, Emory University, Atlanta, Georgia⁶Department of Psychiatry and Behavioral Sciences, Johns Hopkins University, Baltimore, Maryland

Received 9 June 2006; Accepted 20 August 2006

How to cite this article: Maslen CL, Babcock D, Robinson SW, Bean LJH, Dooley KJ, Willour VL, Sherman S. 2006. CRELD1 mutations contribute to the occurrence of cardiac atrioventricular septal defects in down syndrome. Am J Med Genet Part A 9999:1–5.**To the Editor:**

Down syndrome (DS), the most common human autosomal aneuploidy, results from trisomy of chromosome 21. Congenital heart defects occur in approximately 40–50% of DS cases, with the majority being defects in septation [Pradat, 1992; Freeman et al., 1998; Stoll et al., 1998]. Although the spectrum of heart defects observed in DS is varied, the frequency of atrioventricular septal defect (AVSD) is striking. Among children with a normal karyotype, the frequency of AVSD is 1 in 10,000 live births, but in the DS population the frequency is 2,000 in 10,000 live births, or approximately half of all congenital heart defects [Ferencz et al., 1997].

Atrioventricular septal defect (AVSD), also known as an atrioventricular canal defect or endocardial cushion defect, is a congenital cardiac anomaly that occurs when the superior and inferior endocardial cushions fail to close completely, resulting in incomplete formation of the atrial and ventricular valves and septa. In the most severe form, a complete AVSD, there is a hole called an ostium primum atrial septal defect in the lower portion of the atrial septum, a ventricular septal defect in the upper portion of the ventricular septum, and related valve defects. In milder forms, known as partial AVSDs, there is an ostium primum atrial septal defect often occurring with a cleft in the anterior leaflet of the mitral valve. Only patients with the most severe form, a complete AVSD, were included in this study.

Genetic complexity and heterogeneity is a hallmark of AVSD. Familial cases of isolated AVSD with clear monogenic, autosomal dominant transmission have been reported; however, they are the exception since only 5–10% of isolated AVSD have an affected first degree family member [Emanuel et al., 1983; Ferencz et al., 1997; Digilio et al., 1999]. The sporadic occurrence of isolated AVSDs suggests that AVSD is either a complex trait, influenced by both genetic and environmental susceptibility factors, or a monogenic trait caused by a high mutation rate in one of a few AVSD genes [Maslen, 2004].

Two specific genetic loci for AVSD have been identified. The *AVSD1* locus on chromosome 1p31-p21 (OMIM 606215) was delineated through characterization of a family with autosomal dominant AVSD with incomplete penetrance [Sheffield et al., 1997]. However, the *AVSD1* gene itself remains unknown. The *AVSD2* locus on chromosome 3p25 (OMIM 606217) was defined by breakpoints in the

Grant sponsor: PHS; Grant number: 5 M01 RR00334; Grant sponsor: NIH; Grant numbers: P01 HD24605, R01 HD38979, F32 HD046337; Grant sponsor: Children's Healthcare of Atlanta Cardiac Research Committee; Grant sponsor: CRC US DHS NIH; Grant number: MO1 RR00039.

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Published online 00 Month 2006 in Wiley InterScience (www.interscience.wiley.com)

DOI 10.1002/ajmg.a.31494

rare cytogenetic disorder known as 3p-syndrome [Phipps et al., 1994; Green et al., 2000]. There are also numerous syndromes that include AVSD as a phenotypic component [for a comprehensive list see Lin et al., 2006]. Of note, trisomy 21 is by far the most common finding associated with AVSD.

Given the incidence of congenital heart defects in DS there has been a great deal of focus on chromosome 21 as a source for congenital heart defect susceptibility genes, including those involved in AVSD. Many studies have been based on the premise that increased expression of chromosome 21 genes causes the features of DS, including congenital heart defects [Deutsch et al., 2005; Mao and Pevsner, 2005; Li et al., 2006]. Attempts to identify dosage sensitive chromosome 21 genes that contribute to heart defects have focused on analysis of individuals with segmental trisomy or monosomy 21 to identify “critical regions,” the smallest regions of chromosome 21 overlap between individuals who share a DS-associated phenotype. Resolution using this approach is limited due to the rarity of the condition, the complex karyotype of such individuals, which frequently includes more anomalies than segmental trisomy, and to the heterogeneity of the defined phenotype. For example, the “heart defect critical region” which extends from 21q22.13 to 21qter between markers *D21S55* and *COL6A2* is based on seven patients with a variety of heart defects [Korenberg et al., 1992]. A narrowed heart critical region was proposed by Barlow et al. [2001] that excluded several genes distal to *D21S55* including the collagen genes, *COL6A1*, *COL6A2*, and *COL18A*, which other studies suggest are important candidates for heart defects in DS [Davies et al., 1994, 1995]. To date no single gene or set of genes on chromosome 21 has been shown to contribute to the risk of heart defects in DS or euploid individuals.

We previously identified *CRELD1* as a candidate gene for the *AVSD2* locus on chromosome 3p25 based on physical mapping to that locus and studies demonstrating expression in the developing AV endocardial cushions [Rupp et al., 2002]. *CRELD1* encodes a cell surface protein that likely functions as a cell adhesion molecule. A subsequent study of 50 individuals with AVSD identified three missense mutations in *CRELD1* that are specifically associated with AVSD [Robinson et al., 2003]. Analysis of DNA from family members of one of the probands with a partial AVSD in which a missense mutation (p.R329C) was identified showed that the mutation was inherited from the father. The sister of the proband also carried the mutation. Two-dimensional echocardiography and color flow Doppler studies indicated that the father and sister had structurally and functionally normal hearts, demonstrating incomplete penetrance of this *CRELD1* variant. In a later study Zatyka et al. [2005] identified another missense mutation in an individual with a partial

AVSD that also was incompletely penetrant. Together these results suggest that *CRELD1* mutations act in a dominant manner with incomplete penetrance and that *CRELD1* is an AVSD susceptibility gene that may act in concert with additional modifier genes at other loci.

A separate study by Sarkozy et al. [2005] found no pathogenic mutations in an additional 31 individuals with isolated AVSD. Collectively, patient studies indicate that *CRELD1* mutations occur in about 3% of euploid individuals with AVSD, most frequently in cases of partial AVSD. About 4.5% of individuals with partial AVSD have a *CRELD1* mutation.

Given that *CRELD1* mutations appear likely to be genetic risk factors for AVSD in the euploid population, we hypothesized that mutations in this gene may contribute to the occurrence of AVSD on the genetic background of trisomy 21. To test this hypothesis we resequenced *CRELD1* in 39 individuals with DS and a complete AVSD, which is the most common heart defect associated with DS. Thirty two of these individuals were ascertained through a larger study of DS aimed at understanding the cause and resulting phenotype of trisomy 21 [Freeman et al., 1998; Kerstann et al., 2004]. An additional seven individuals were ascertained from the Sibley Heart Center, Cardiology, Children’s Healthcare of Atlanta specifically because they had DS and a complete AVSD. Twenty three of the subjects were ascertained at less than 1 year of age. The remaining subjects ranged in age from 1 to 15 years of age (mean = 5.06 years, median = 4 years). Blood samples were obtained from the person with DS and their parents. Questionnaires were completed by the mother and father to obtain demographic information about the family as well as reproductive histories, maternal and paternal health histories, and environmental exposures. Medical records on the affected individual were abstracted using a structured form to obtain information about the heart defect as well as other abnormalities. Echocardiograms and surgical reports were required to definitively diagnose complete AVSD. For this preliminary study, we included only self-reported non-Hispanic Caucasians. All participants were enrolled under a protocol that was approved by human subjects committees at all recruitment sites. Genomic DNA was provided as coded samples to the Maslen laboratory for mutation analyses.

Patient genomic DNA was analyzed for variation in the *CRELD1* gene by resequencing, as previously described [Robinson et al., 2003]. Briefly, overlapping PCR amplicons encompassing the entire *CRELD1* coding region of 11 exons, including all intron–exon boundary junctions and at least 100 bp of intron covering potential splicing elements, such as the branch point sites, were generated as templates for DNA sequence analysis. The templates were sequenced in both directions by the OHSU General

Clinical Research Center DNA Sequencing Facility. The sequencing electropherograms were analyzed (by C.L.M, D.B, and S.W.R) using MutationSurveyor™ DNA Analysis software. All base changes detected were heterozygous. All alterations were confirmed by allele-specific PCR analysis as previously described [Maslen et al., 1997; Babcock et al., 1998; Robinson et al., 2003]. The National Center for Biotechnology database of genetic variation (dbSNP) was queried to identify DNA sequence alterations that were commonly occurring SNPs. Population studies were performed for alleles that appeared to be unique (described below). Apparently unique alleles were confirmed in a second aliquot of DNA from the study subject, provided as an independent sample. DNA from the parents of the subjects was also analyzed for the presence of the mutant allele.

We identified two heterozygous missense mutations in two unrelated subjects with DS and AVSD. One was an infant that carried a recurrent mutation originally identified in an unrelated young adult with a sporadic, isolated partial AVSD (ostium primum ASD) [Robinson et al., 2003]. The infant in the current study also had a secundum atrial septal defect, patent ductus arteriosus, and tricuspid regurgitation. Of note, there was anomalous hepatic drainage to the right atrium. This patient also had pulmonary hypertension. No other anomalies except for those common in DS were noted.

The mutation is a C to T transition in exon 9 at cDNA position 985 (c.985C > T) that results in a substitution of cysteine for arginine at amino acid 329 (p.R329C) in the second calcium binding-EGF domain of the protein [Rupp et al., 2002]. The recurrent nature of the mutation is likely due to occurrence at a CpG dinucleotide, creating a mutation hotspot. The mutation was inherited from the mother, a normal euploid individual with no evidence of a heart defect.

This is consistent with our previous data that showed incomplete penetrance for p.R329C.

We previously determined that the p.R329C alteration was not present in 400 race-relevant control chromosomes [Robinson et al., 2003], which has the power to detect a ≥1% polymorphism with 95% confidence [Collins and Schwartz, 2002]. In the current study we also examined DNA from 30 individuals (60 chromosomes) with trisomy 21 and absence of congenital heart defects as documented by echocardiography. None of these “controls” carried the p.R329C mutation. In fact, we do not expect the frequency of *CRELD1* mutations to differ between euploid and DS controls since there is no known relationship between *CRELD1* and non-disjunction events resulting in trisomy 21 that would alter the allele distribution in that population.

Calcium binding EGF domains are highly conserved with specific disulfide bonding patterns. Consequently, addition of a free cysteine residue, as occurs with this mutation, would be expected to interfere with protein folding. As expected, the p.R329C mutation alters the protein structure as was previously shown in an analysis of recombinantly expressed mutant *CRELD1* [Robinson et al., 2003]. Furthermore, this amino acid position is conserved as an arginine residue among mammals (Fig. 1A). Taken together, these data suggest a specific association of the p.R329C mutation with AVSD. The severity of the heart defect was greater in the individual with DS, who had a complete AVSD and additional cardiac anomalies, compared to the affected euploid individual with an isolated partial AVSD, raising the possibility that trisomy 21 exacerbates the effect of the *CRELD1* C329 allele.

The second mutation was identified in an infant with DS and a complete balanced AVSD and tricuspid regurgitation. There were no additional



Fig. 1. **A:** Alignment of the sequence for the cb-EGF domain encoded by exon 9 from the human, bovine, dog, rat, and mouse *CRELD1* genes. **B:** Alignment of the amino acid sequence encoded by exon 10 from the human, mouse, and bovine *CRELD1* genes. An asterisk beneath the sequences denotes amino acid residues that are identical between these species. The amino acid residues changed by the missense mutations in humans are boxed.

unexpected cardiac or extracardiac anomalies noted. The alteration was a G to A transition in exon 10, c.1240G>A, resulting in the non-conservative substitution of a lysine for a glutamic acid residue at amino acid position 414 (p.E414K). We used allele-specific PCR analysis to assay 400 race-relevant control chromosomes and found that the p.E414K mutation was not present in these controls. It was also absent from 60 chromosomes of individuals with DS and no heart defect. Both parents of this proband were normal euploid individuals. Neither parent had clinical evidence of a heart defect, although no echocardiograms were conducted. The mutation was not detected in either parent, indicating that it occurred de novo in the proband, or was a germline mutation in one of the parents. The substitution of a lysine for a glutamic acid residue introduces a change in charge at a position that is highly conserved among mammals (Fig. 1B). Indeed, the only substitution at that position in more distantly related species is a conservative change to an aspartic acid residue, indicating that an acidic residue is optimal in that position. Figure 2 illustrates the position of all AVSD-associated *CRELD1* mutations identified to date.

Identification of *CRELD1* mutations in 2/39 individuals (5.1%) with DS and complete AVSD suggests that defects in *CRELD1* may contribute to the pathogenesis of AVSD in the context of trisomy 21. This is consistent with our previous study that demonstrated that *CRELD1* mutations were associated with AVSD and heterotaxy as well as non-syndromic AVSD. Consequently we conclude that *CRELD1* mutations in general increase risk for developing an AVSD, with other factors such as trisomy 21 and environmental factors further weighting the balance in that direction. These mutations may be inherited or occur de novo.

The question remains as to what combination(s) of factors is required to exceed the threshold causing a complete AVSD. Individuals with DS have a 2,000-fold increased occurrence of complete AVSD

[Ferencz et al., 1989], making this the most highly sensitized population to congenital heart defects. Our working model to account for the greatly increased incidence of congenital heart disease in DS is that trisomy 21 takes the place of multiple modifiers, so that fewer additional predisposing mutations are required to reach a heart defect in a person with trisomy 21. Consequently, the DS population provides a valuable resource for the identification and characterization of additional susceptibility genes that contribute to congenital heart defects, one of the most frequent congenital anomalies among all live births.

ACKNOWLEDGMENTS

Supported in part by PHS grant 5 M01 RR00334 (C.L.M), NIH P01 HD24605 (V.L.W, L.J.H.B), NIH R01 HD38979 (L.J.H.B, S.L.S), F32 HD046337 (L.J.H.B), Children's Healthcare of Atlanta Cardiac Research Committee (L.J.H.B, K.J.D), and CRC US DHS NIH MO1 RR00039 (L.J.H.B, S.L.S). The authors thank Dr. Kim Kerstann and Dr. Rob Pyatt for their contributions to this project, and Dr. Roger Reeves for his helpful comments and guidance.

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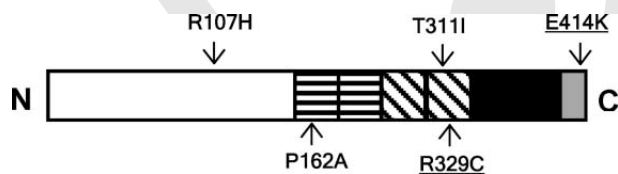
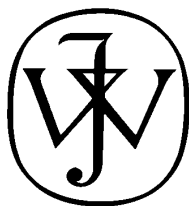


FIG. 2. A diagrammatic representation of *CRELD1* indicating the position and identity of all known AVSD-associated mutations (white, WE domain; horizontal hatching, EGF domains; diagonal hatching, calcium binding EGF domains; black, two pass transmembrane domain; gray, carboxyl-terminal domain). The mutations that were found in individuals with DS + AVSD are underlined. The R107H mutation was found in an individual with an unbalanced AVSD and heterotaxy, the T311I substitution is from an individual with isolated sporadic partial AVSD, and the p.R329C mutation was originally reported in an individual with an isolated sporadic partial AVSD [Robinson et al., 2003] and has now been detected in an individual with DS + AVSD. The P162A substitution was recently reported for an individual with a sporadically occurring, isolated complete AVSD [Zatyka et al., 2005].

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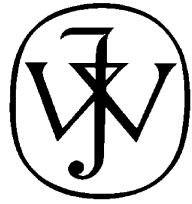
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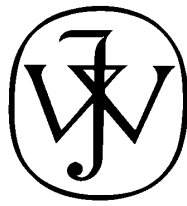
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