Novel *CRELD1* gene mutations in patients with atrioventricular septal defect

Ying Guo, Jie Shen, Lang Yuan, Fen Li, Jian Wang, Kun Sun

Shanghai, China

Background: **Atrioventricular septal defects (AVSDs) occur as clinical defects of several different syndromes, as autosomal dominant defects, and as sporadically occurring malformations. Consequently, there is genetic heterogeneity, but until recently, little is known about the genes involving in the pathogenesis of AVSD. *CRELD1* gene, a novel cell adhesion molecule, is a candidate gene for AVSD.**

Methods: **This study included 133 patients with AVSD and 200 healthy controls. Peripheral blood samples were collected and genomic DNA was extracted from the leukocytes. *CRELD1* was amplified by polymerase chain reaction (PCR) with specific primers. The sequences of PCR products were compared between the patients and controls.**

Results: **In a patient, a C-to-G transition was identified at nucleotide 857 in exon 8 that resulted in a substitution of alanine for proline at amino acid 286 in the first calcium-binding EGF domain. This patient had an isolated partial AVSD and the mutation was inherited from her mother. Another mutation was detected in a patient with a partial AVSD and evidence of Down syndrome. The heterozygous c.973G>A transition in exon 9 resulted in a substitution of lysine for glutamic acid at amino acid 325 (E325K) in the second calcium-binding EGF domain.**

Conclusions: **Two novel *CRELD1* mutations were identified in the calcium-binding EGF domain in patients with AVSD. *CRELD1* is likely to be an AVSD-susceptibility gene and *CRELD1* mutations may increase the risk of developing a heart defect rather than being a direct causative mutation.**

World J Pediatr 2010;6(4):348-352

Key words: **atrioventricular septal defect; *CRELD1*; Down syndrome**

Introduction

Atrioventricular septal defect (AVSD), also known as atrioventricular canal defect or endocardial cushion defect, is a subset of malformation that constitutes 7%-8% of all recognized congenital heart diseases.[1] AVSD is sometimes referred to as an endocardial cushion defect because failure of endocardial cushion formation results in incomplete valvuloseptal morphogenesis. The atrioventricular endocardial cushion contributes to the formation of the inlet ventricular septum and atrial septum primum and the mitral and tricuspid valves. Consequently, genes involving in the development of the atrioventricular endocardial cushions have been recognized as prime candidates for AVSD.

To the present, two specific genetic loci for AVSD have been identified. The study of one large family led to the identification of the AVSD1 locus on chromosome 1p31-p21 (MIM 606215) by use of a combination of DNA pooling and shared segment analysis in a high-density genome screening.[2] Although the responsible gene has not yet been identified, this study demonstrated the existence of a congenital heart susceptibility gene inherited as an autosomal dominant trait with incomplete penetrance. In another study, analysis of chromosomal breakpoints in 3p-syndrome found the AVSD2 (MIM 606217) locus on chromosome 3p25.[3] The corresponding gene is *CRELD1* (GenBank accession number AF452623), which belongs to an epidermal growth factor-like family and encodes a cell surface protein that likely functions as cell adhesion molecule. *CRELD1* encodes a novel cell adhesion molecule that is expressed during cardiac cushion development.[4] To further investigate the role of *CRELD1* in AVSD

Author Affiliations: Department of Cardiology, Shanghai Children's Medical Center, School of Medicine, Shanghai Jiao Tong University, Shanghai 200127, China (Guo Y, Li F, Wang J); Department of Cardiology, Shanghai Children's Hospital, Shanghai Jiao Tong University, Shanghai 200040, China (Shen J, Yuan L); Department of Pediatric Cardiology, Xinhua Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200092, China (Sun K)

Corresponding Author: Kun Sun, MD, PhD, Department of Pediatric Cardiology, Xinhua Hospital, 1665 Kongjiang Road, Shanghai 200092, China (Fax: +86-21- 65125173; Email: drkunsun@yahoo.com.cn)

doi:10.1007/s12519-010-0235-7

pathogenesis, we analyzed 133 patients with AVSD for *CRELD1* mutations who had been admitted to Shanghai Children's Medical Center from March 2007 to October 2009.

Methods

All participants (133 patients and 200 healthy controls) of this study were of the Chinese Han nationality. Blood samples were taken from the 133 patients aged from 2 months to 12 years. Clinical examination of the patients included anthropometric measurement, physical examination for dysmorphism and malformation, chest X-ray examination, electrocardiography, ultrasonic echocardiography, and some of the patients even underwent cardiac catheterization examination. The anatomic phenotypes of the patients are shown in Table 1. Among the 133 patients, 24 had Down syndrome, 2 had heterotaxy syndromes and polysplenia, 6 had heterotaxy syndromes and asplenia, and 101 had non-syndrome related AVSD. In 95 patients with complete AVSD, 35 were associated with other congenital heart abnormalities. Thirty-eight patients with partial AVSD were associated with clefting of the mitral valve. DNA samples from 200 unrelated and healthy individuals of the Chinese Han nationality (93 males and 107 females) served as controls. Before the study, informed consents were obtained from their parents or guardians of both patients and controls. The research protocols and procedures were approved by the Association of Medical Ethics of Shanghai Children's Medical Center.

Peripheral blood samples were collected, and genomic DNA of each sample was extracted by use of Axygen DNA extraction kits (Axygen, Union City, CA). Primers were designed to amplify 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 (Table 2). Polymerase chain reaction (PCR) products from each exon were tested and directly sequenced according to the ABI Big Dye Terminator Cycle Sequencing protocol and ABI 310 Automated Sequencer (ABI, Foster City, CA, USA).

BLAST search was used to identify homology between the sequences obtained from patients and to evaluate sequence conservation across species.

Table 1. The anatomic phenotypes of the 133 patients included in the molecular screening

Type of congenital heart defect	*n*	
Non-DS complete AVSD	73	
Isolated	41	(one with cleft, and one patient's father with SAS)
With LSVC	2	
With PDA	3	
With DORV	5	
With SV	9	
With PA/VSD	5	
With DORV and heterotaxy	8	
Complete AVSD with DS	22	
Isolated	19	
With PDA	3	
Non-DS partial AVSD	36	
Isolated	35	
With PDA	1	
Partial AVSD with DS	2	
Isolated	2	
Total	133	

DS: Down syndrome; LSVC: left superior vena cava; PDA: patent ductus arteriosus; DORV: double outlet right ventricle; SV: single ventricle; PA/VSD: pulmonary atresia and ventricular septal defect; SAS: supravalvar aortic stenosis.

Table 2. Primers for the amplification of *CRELD1* exons

Exons	Forward primer	Reverse primer	Amplicon (bp)
1	5′- CCTCTTTTGCTTGTAATAACGCAG	5′- CCACTCTAAATCGCTGTGTTCC	361
2	5′- GGGAACACAGCGATTTAGAGTGG	5′- ACCAGCAGAGCAGAGATTTGGC	341
3	5′- AGGAAGGGTGGAGAGAGACTTGAG	5′- TGGGTTTGGAAGGAGCACAAC	318
4	5′- GGGAATGGGAACAGCACTTATGAC	5′- GGCAAGAACTGAGGTCCAGAGAAG	297
5	5′- TTGGTGTCTTCCCACAGCCT	5′- TGCAAACAGTTTGATTCCTCAG	333
6	5′- TTGGCTACTTTGAGGCAGAACG	5′- CCCATCACCACTTCCACTGAAC	468
7	5′- ATCCCAGGCAAGACCATTCC	5′- CAGCTCTTCCTCCACTCCTCA	226
8	5′- CCTGTCCCTCCAAACCTTCC	5′- CCCAGCACCCTGACTCCAT	383
9	5′- AGAGGGGAGTGTTGAGAGATGGAC	5′- CTGATTTGATGCCAGGTCTGATTC	446
10	5′- CGGGCTCTACATCTGATCTCC	5′- AGAGGTAAAGGGGTTGGACTTG	510
11	5′- TGTGTGGGAGTTCTGGGGAGAC	5′- TACCTGGGCTGCTCTGTAAGGC	441

Table 3. SNPs detected in *CRELD1*

SNP	Location	Aa change	Frequency in atrioventricular septal defect cases	Frequency in control subjects	rs number
C1119T	Exon 10	H373H	39:133	41:200	rs 3774207
C383G	Exon 4	P128R	18:133	18:200	rs 2302787

Results

A heterozygous c.857C>G mutation was identified in one patient with isolated partial AVSD (Fig. 1). The mutation resulted in a substitution of arginine for proline at amino acid 286 (P286R) in the first calcium-binding EGF domain. After exclusion in 200 normal chromosomes of the Chinese population we considered the variant to be a novel *CRELD1* mutation in patients with AVSD. Analysis of genomic DNA from the parents of the proband showed that this mutation was inherited from the mother. Two-dimensional echocardiography and color flow Doppler studies showed that the mother had structurally normal heart with normal ventricular function, demonstrating incomplete penetrance of this *CRELD1* variant.

The second mutation was identified in a patient with Down syndrome, a partial AVSD and pulmonary hypertension. The heterozygous c.973G>A transition in exon 9 resulted in a substitution of lysine for glutamic acid at amino acid 325 (E325K) in the second calcium-binding EGF domain (Fig. 2). The parents of the proband refused to do genetic analysis. The variant was not detected in 200 normal chromosomes of the Chinese population.

Cross-species analysis demonstrated that these nucleotides and codons were conserved: >90% homology in regions of the *CRELD1* gene (Fig. 3). In addition, we detected two previously reported polymorphisms (rs 2302787 and rs 3774207) (Table 3).

Discussion

AVSDs include a spectrum of anomalies of the atrioventricular valves and the atrial and ventricular septa. In the complete form there are a single common atrioventricular valve, an atrial septal defect (ostium primum), and a confluent posterior ventricular septal defect in the inlet portion of the ventricular septum. Without intervention, few patients survive to 5 years of age due to the early onset of heart failure and irreversible pulmonary hypertension. In the partial form there are two separate right and left atrioventricular valves with a "cleft" of the mitral valve, an atrial septal defect (ostium primum), and no ventricular septal communication.

From the pathogenetic point of view, this malformation is classified in a group of defects of the extracellular matrix.[5-7] The atrioventricular septal defect is a "classic" heart defect in children with Down syndrome and is one of the congenital heart diseases frequently associated with extracardiac anomalies.[8-11] The evident genetic basis for this malformation is demonstrated also by the prevalence of patients with heterotaxy, other chromosomal defects or Mendelian syndromes.[10,11] Based on anatomical, clinical, and genetic observations,[7,12-14] four major groups of patients with atrioventricular canal defect have been recognized: group I, Down syndrome (45%); group II, heterotaxy syndrome (15%); group III, other syndromes with situs solitus (15%), such as 3p-syndrome, CHARGE syndrome; and group IV,

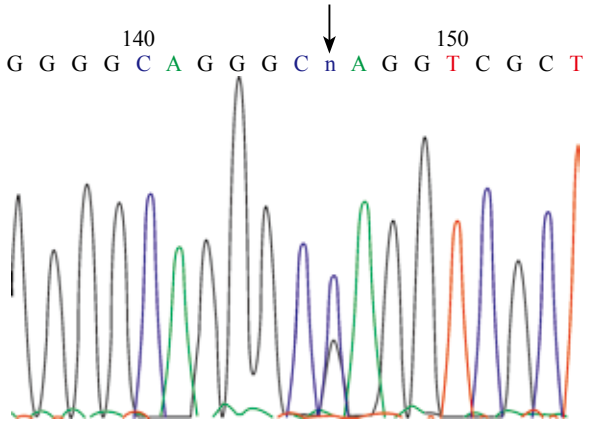

Fig. 1. Sequence of the proband (C857G missence mutation).

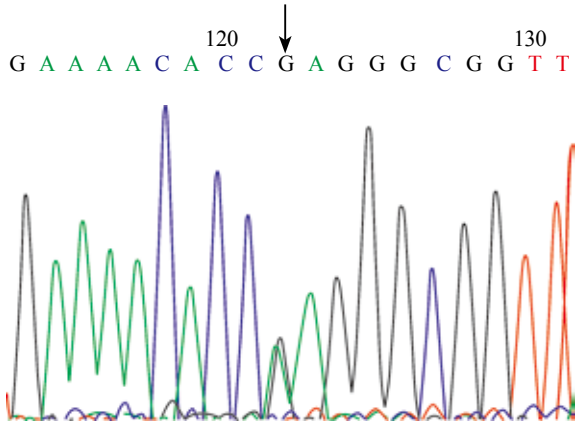

Fig. 2. Sequence of the proband (G973A missence mutation).

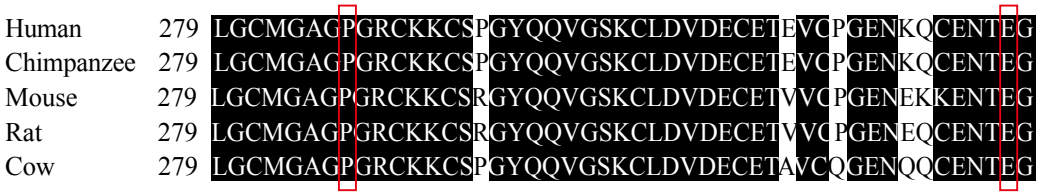

Fig. 3. Alignments of the known *CRELD1* homologues.

nonsyndromic patients (25%). Different genetic bases could explain the anatomical differences in the various genetic groups of patients with AVSD. Several families have been found to have autosomal-dominant AVSD with incomplete penetrance,[15,16] demonstrating that AVSD can be inherited as a single gene defect. But the majority cases (about 65%) of AVSD is sporadic.[11] The relatively high incidence of sporadic cases indicates that AVSD is usually genetically complex, with multiple factors contributing to susceptibility and variability of expression.

The *CRELD1* family consists of two matricellular proteins thought to be involved in cell adhesion processes. Identification and characterization of the *CRELD1* gene on chromosome 3p25 led to the establishment of *CRELD1* as the first known genetic risk factor for isolated AVSD.

In 52 patients with non-Down syndrome-related AVSD, Robinson et al[17] identified 3 *CRELD1* mutations (p. R107H, p.T311I, p.R329C) in those patients with partial AVSD. Zatyka et al[18] identified another missense mutation (p. P162A) in an individual with partial AVSD. Sarkozy et al[19] found no pathogenic mutations in 31 individuals with isolated AVSD. But Posch et al[20] did not identify any mutations in 16 AVSD patients. Our studies and previous reports of *CRELD1* gene screening showed that the incidence of *CRELD1* gene mutations in non-Down syndrome-related AVSD is 2.0% (5/253 patients analyzed). In the non-Down syndrome group, *CRELD1* mutations were detected exclusively in patients with partial AVSD (5/121; 4.1%; not including Posch's cases), suggesting a genotype-phenotype correlation with this specific anatomic AVSD subtype. In our study, the proband inherited the *CRELD1* P286R mutation from the mother who had a structurally and functionally normal heart. As reported previously, these findings are consistent with incomplete penetrance.[21] The presence of unaffected family members carrying a *CRELD1* mutation showed that *CRELD1* mutation is neither necessary nor sufficient to cause AVSD, indicating that mutation of *CRELD1* increases susceptibility for AVSD rather than serves as a monogenic cause.

AVSD is commonly associated with Down syndrome. Among children with a normal karyotype, the frequency of AVSD is 1 in 10 000 live births, but is 2000 in 10 000 live births in the Down syndrome population.[22] The study of the relationship between *CRELD1* mutation and the occurrence of AVSD on the genetic background of trisomy 21 was rare. Maslen et al[23] identified 2 heterozygous missense mutations (R329C, E414K) in 2 subjects with Down syndrome and AVSD. One had partial AVSD with a secundum atrial septal defect, patent ductus arteriosus, tricuspid regurgitation and pulmonary hypertension; the other had complete AVSD and tricuspid regurgitation. Interestingly, with the same R329C mutation, the severity of the heart defect was greater in a patient with Down syndrome, who had a complete AVSD and pulmonary hypertension, compared to the patient with an isolated partial AVSD. These data and ours indicate that the incidence of *CRELD1* mutation in patients with AVSD associated with Down syndrome is 4.8% (3/63). There is a significant difference in the frequency of *CRELD1* mutation between patients with Down syndrome and those without Down syndrome (χ^2=1.586, *P*=0.208). The results indicate that the mutation of *CRELD1* gene may contribute to the pathogenesis of AVSD in the context of trisomy 21, possibly through genetic interaction with undetermined causative gene(s) on chromosome 21 and environmental factors.

The two novel mutations associated with AVSD occurred in calcium-binding EGF domains. Up to now, 7 *CRELD1* mutation sites associated with AVSD have been identified and 4 of them are in calcium-binding EGF domains (Fig. 4). The calcium-binding EGF domain is prevalent in many extracellular proteins, and heterozygous mutations affecting calcium-binding EGF domains have been associated with a number of genetic disorders. Calcium-binding EGF domains are highly conserved with specific disulfide bonding patterns. The lysine-to-arginine and glutamic acid-to-proline substitutions in calcium-binding EGF domains, the change of the amino acid polarity, would be expected to interfere with protein folding and further affect the protein function.

In conclusion, the present mutation analysis of *CRELD1* gene in 133 Chinese AVSD patients revealed 2 novel mutations in the calcium-binding domain. To our knowledge, the sample size in our study is the largest. The overall mutation rate was low (2/133, 1.5%) and unaffected family members carrying a *CRELD1* mutation indicate that *CRELD1* is an AVSD-

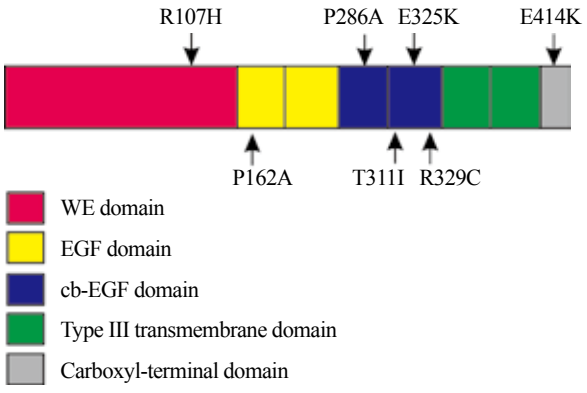

Fig. 4. Diagrammatic representation of all known AVSD-associated mutations.

susceptibility gene and that *CRELD1* mutations increase the risk of developing a heart defect rather than serve as direct causative mutations.

Acknowledgements

We are grateful to the patients and families for their participation in this study.

Funding: This work was supported by grants from the National Natural Science Foundation of China (No. 30772348) and Bang Bao Scholar Program.
Ethical approval: This protocol was approved by the Hospital Ethical Committee.
Competing interest: None.
Contributors: Guo Y wrote the main body of the article. Sun K and Shen J are supervisors of this article. Guo Y and Yuan L contributed equally to this paper. Li F and Wang J provided advice on medical aspects.

References

1 Ferencz C, Loffredo CA, Rubin JD, Magee CA. Perspectives in pediatric cardiology. NY: Futura Publishing Company press, 1997.
2 Sheffield VC, Pierpont ME, Nishimura D, Beek JS, Burns TL, Berg MA, et al. Identification of a complex congenital heart defect susceptibility locus by using DNA pooling and shared segment analysis. Hum Molec Genet 1997;6:117-121.
3 Green EK, Priestley MD, Waters J, Maliszewska C, Latif F, Maher ER. Detailed mapping of a congenital heart disease gene in chromosome 3p25. J Med Genet 2000;37:581-587.
4 Rupp PA, Fouad GT, Egelston CA, Reifsteck CA, Olson SB, Knosp WM, et al. Identification, genomic organization and mRNA expression of *CRELD1*, the founding member of a unique family of matricellular proteins. Gene 2002;293:47-57.
5 Clark EB. Mechanisms in the pathogenesis of congenital heart defects. In: Pierpont ME, Moller J, eds. The Genetics of Cardiovascular Disease. Boston: Martinus-Nijoff, 1986: 3-11.
6 Clark EB. Pathogenetic mechanism of congenital cardiovascular malformations revisited. Semin Perinatal 1996;20:465-472.
7 Marino B, Digilio MC, Toscano A, Giannotti A, Dallapiccola B. Congenital heart diseases in children with Noonan syndrome: an expanded cardiac spectrum with high prevalence of atrioventricular canal. J Pediatric 1999;135:703-706.
8 Ferencz C, Loffredo CA, Correa-Villasenor A, Wilson PD. Genetic and Environmental Risk Factor of Major Cardiovascular Malformations. The Baltimore-Washington Infant Study 1981-1989. New York: Futura Publishing press, 1997.
9 Marino B, Vairo U, Corno A, Nava S, Guccione P, Calabrò R, et al. Atrioventricular canal in Down syndrome. Prevalence of associated cardiac malformations compared with patients without Down syndrome. Am J Dis Child 1990;144:1120-1122.
10 Carmi R, Boughman JA, Ferencz C. Endocardial cushion defect: further studies of "isolated" versus "syndromic" occurrence. Am J Med Genet 1992;43:569-575.
11 Digilio MC, Marino B, Toscano A, Giannotti A, Dallapiccola B. Atrioventricular canal defect without Down syndrome: a heterogeneous malformation. Am J Med Genet 1999;85:140-146.
12 Marino B. Left-sided cardiac obstruction in patients with Down syndrome. J Pediatr 1989;115:834-835.
13 Francalanci P, Marino B, Boldrini R, Abella R, Iorio F, Bosman C. Morphology of the atrioventricular valve in asplenia syndrome: a peculiar type of atrioventricular canal defect. Cardiovasc Pathol 1996;5:145-151.
14 Digilio MC, Marino B, Ammirati A, Borzaga U, Giannotti A, Dallapiccola B. Cardiac malformations in patients with oral-facial-skeletal syndromes: clinical similarities with heterotaxia. Am J Med Genet 1999;84:350-356.
15 Cousineau AJ, Lauer RM, Pierpont ME, Burns TL, Ardinger RH, Patil SR, et al. Linkage analysis of autosomal dominant atrioventricular canal defects: exclusion of chromosome 21. Hum Genet 1994;93:103-108.
16 Wilson L, Curtis A, Korenberg JR, Allan L, Chenevix-Trench G, Stephenson A, et al. A large, dominant pedigree of atrioventricular septal defect (AVSD)-exclusion from the Down syndrome critical region on chromosome 21. Am J Hum Genet 1993;53:1262-1268.
17 Robinson SW, Morris CD, Goldmuntz E, Reller MD, Jones MA, Steiner RD, et al. Missense mutations in *CRELD1* are associated with cardiac atrioventricular septal defects. Am J Hum Genet 2003;72:1047-1052.
18 Zatyka M, Priestley M, Ladusans EJ, Fryer AE, Mason J, Latif F, et al. Analysis of *CRELD1* as a candidate 3p25 atrioventicular septal defect locus (AVSD2). Clin Genet 2005;67:526-528.
19 Sarkozy A, Esposito G, Conti E, Digilio MC, Marino B, Calabrò R, et al. *CRELD1* and *GATA4* gene analysis in patients with nonsyndromic atrioventricular canal defects. Am J Med Genet A 2005;139:236-238.
20 Posch MG, Perrot A, Schmitt K, Mittelhaus S, Esenwein EM, Stiller B, et al. Mutations in GATA4, NKX2.5, *CRELD1*, and BMP4 are infrequently found in patients with congenital cardiac septal defects. Am J Med Genet 2008;146A:251-253.
21 Pierpont ME, Markwald RR, Lin AE. Genetic aspects of atrioventricular septal defects. Am J Med Genet 2000;97:289-296.
22 Ferencz C, Boughman JA, Neill CA, Brenner JI, Perry LW. Congenital cardiovascular malformations: questions on inheritance. Baltimore-Washington Infant Study Group. J Am Coll Cardiol 1989;14:756-763.
23 Maslen CL, Babcock D, Robinson SW, Bean LJ, Dooley KJ, Willour VL, et al. *CRELD1* mutations contribute to the occurrence of cardiac atrioventricular septal defects in Down syndrome. Am J Med Genet A 2006;140:2501-2505.

Received April 20, 2010
Accepted after revision August 26, 2010