Molecular Genetic Analysis of CRELD1 in Patients with Heterotaxy Disorder

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Molecular Genetic Analysis of *CRELD1* in Patients with Heterotaxy Disorder

by

Samaneh Zhian

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science
in
Biology

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ABSTRACT

Heterotaxy refers to the abnormal arrangement of internal organs in relation to each other. Model organism studies have shown that functions of more than eighty genes are required for normal asymmetric left-right organ development. *CRELD1* has been shown to be necessary for proper heart development and mutations in *CRELD1* are known to increase risk of cardiac atrioventricular septal defects (AVSD). AVSD is the most common form of heart defect associated with heterotaxy, and we have previously shown that some individuals with heterotaxy-related AVSD have mutations in *CRELD1*.

Therefore, we propose to examine the *CRELD1* gene in a large sample of patients with heterotaxy syndrome. Our goal was to determine if mutations in *CRELD1* are associated with other manifestations of heterotaxy or if they only coincide with AVSD. To achieve this aim, a sample size of 126 patients with heterotaxy collected by Dr. Belmont, Baylor college of Medicine, Texas, with approximately 66% of the heterotaxy population with different types of heart defects, were used for this study. Ten exons, promoter regions, and regulatory elements in the introns of *CRELD1* gene were sequenced and analyzed.

In this study three different heterozygous missense mutations in *CRELD1* were identified in three unrelated individuals. These three individuals were diagnosed with different forms of heart defects in addition to AVSD. All three mutations were identified in highly conserved regions of *CRELD1* possibly altering the CRELD1 properties. This demonstrates that mutations in *CRELD1* may increase the
susceptibility of AVSD in heterotaxy population. This information can help us to find factors effecting disease susceptibility in heterotaxy patients since the heart defects are a complex trait with incomplete penetrance.
Dedication

This thesis is dedicated to my dear parents, who offered their unconditional love and support throughout this adventure and always encourage me to go through challenges.
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TABLE OF CONTENTS

ABSTRACT ................................................................................................................................. i
DEDICATION ................................................................................................................................. iii
ACKNOWLEDGEMENTS ................................................................................................................ iv
LIST OF TABLES ......................................................................................................................... vii
LIST OF FIGURES ....................................................................................................................... viii
LIST OF ABBREVIATIONS .......................................................................................................... ix

CHAPTER I: INTRODUCTION ........................................................................................................ 1
  1.1-HETEROTAXY AND PHENOTYPIC CHARACTRISTICS ......................................................... 1
  1.2-EPIDEMOIOLOGY AND INHERITANCE OF HETEROTAXY AND OTHER DISORDERS OF L-R ASYMMETRY ................................................................. 3
  1.3-L-R AXIS ESTABLISHMENT DURING EMBRYONIC DEVELOPMENT .................................. 5
  1.4-CLINICAL AND PRENATAL EVALUATION OF HETEROTAXY ........................................ 9
    1.4.1-THE CLINICAL EVALUATION OF HETEROTAXY ....................................................... 9
    1.4.2-PRENATAL EVALUATION ......................................................................................... 11
  1.5-GENETIC RISK FACTORS AND MOLECULAR DIAGNOSTICS ........................................ 12
    1.5.1-ZINC FINGER PROTEIN ZIC3 ..................................................................................... 12
    1.5.2-NODAL, LEFTYA, CRYPTIC, AND ACVR2B ............................................................. 14
    1.5.3-OTHER RISK FACTORS ............................................................................................. 17
  1.6-CRELD1 AS A CANDIDATE GENE ..................................................................................... 17
  1.7-HYPOTHESIS ..................................................................................................................... 21

CHAPTER II: METHOD AND MATERIALS .................................................................................... 22
  2.1-CLINICAL EVALUATION AND SAMPLING ................................................................... 22
  2.2-DESIGN OF Oligonucleotide Primers ............................................................................ 22
  2.3-PCR AMPLIFICATION ....................................................................................................... 23
  2.4-PURIFICATION OF PCR PRODUCTS .............................................................................. 24
  2.5-SEQUENCE ANALYSIS ................................................................................................. 25
  2.6-ALLELE-SPECIFIC PCR ................................................................................................. 26
  2.7-POLYMORPHISM PHENOTYPING (POLYPHEN-2). ....................................................... 27
CHAPTER III: RESULTS ............................................................................................................. 28
  3.1-PATIENT1-A206T MUTATION ......................................................................................... 29
  3.2-PATIENT2-T265A MUTATION ......................................................................................... 32
  3.3-PATIENT3-R329C MUTATION ......................................................................................... 35
  3.4-OTHER SIGNIFICANT VARIANTS IN CRELD1 .......................................................... 36
CHAPTER IV: DISCUSSION .................................................................................................... 39
REFERENCES .................................................................................................................................. 47
APPENDIX: CRELD1 GENOMIC SEQUENCE ......................................................................... 57
LIST OF TABLES

Table 1.1: Congenital anomalies associated with heterotaxy ............................................... 2
Table 2.1: Oligonucleotide primers for CRELD1 exon amplication by PCR .................. 24
Table 2.2: Template quantities for 1Xcycle-sequencing run.............................................. 25
Table 2.3: Oligonucleotide primers for CRELD1 exon sequencing ............................... 25
Table 2.4: ASP primer sequence ......................................................................................... 26
Table 3.1: The frequency of different abnormalities found in heterotaxy population of study ........................................................................................................................................ 29
Table 3.2: Non-synonymous variants in CRELD1 found in heterotaxy patients .......... 38
LIST OF FIGURES

Figure 1.1: Overview of L-R asymmetry in mouse embryo ........................................... 6

Figure 1.2: Mice embryo at the developmental stage...................................................... 20

Figure 3.1: CRELD1 protein and the relative position of amino acids ......................... 30

Figure 3.2: Sequence analysis identifying first missense mutation in CRELD1........... 31

Figure 3.3: Diagrammatic representation of CRELD1 protein ..................................... 32

Figure 3.4: Illustration of different heart defects............................................................ 33

Figure 3.5: Sequence analysis identifying second missense mutations in CRELD1 .... 34

Figure 3.6: Sequence analysis identifying third missense mutations in CRELD1 ....... 36

Figure 4.1: Approximate ratio of heterotaxy patients diagnosed with heart defects and without heart defects ................................................................. 39

Figure 4.2: Alanine and threonine chemical structure ................................................. 41

Figure 4.3: Arginine and cysteine chemical structure .................................................. 43

Figure 4.4: Diagrammatic representation of the two cb-EGF domain of CRELD .... 45
LIST OF ABBREVIATIONS

A-P: Anterior-posterior
ALK: Activin-like receptors
ASD: Atrial septal defects
ASP: Allele specific PCR
AVSD: Atrioventricular septal defects
BBS: Bardet-Biedl syndrome
CHD: Congenital heart defects
CoA: Coarctation of the aorta
CRELD: Cysteine rich with EGF like domains
D-V: Dorsal-ventral
DILV: Double inlet left ventricle
DMSO: Dimethyl sulfate
dNTP: Deoxynucleotide triphosphate
EGF: Epidermal growth factor
FGF: fibroblast growth factor
L-R: Left-right
LPM: Lateral plate mesoderm
MGA: Malposition of great arteries
PCD: Primary ciliary dyskinesia
PCR: Polymerase chain reaction
SNP: Single nucleotide polymorphism
TGA: Transposition of great artery

TGF: Transcription growth factor

cb-EGF: Calcium binding-EGF

VCFS: Velo-Cardio-Facial syndrome

VSD: Ventral septal defects

ZFDs: Zinc finger domains
CHAPTER I: INTRODUCTION

1.1-**Heterotaxy and phenotypic characteristics**

Heterotaxy (*hetero* meaning ‘other’ and *taxy* meaning ‘arrangement’) refers to an abnormal developmental condition characterized by randomized arrangement of the thoracic and/or abdominal visceral organs, including the heart, lungs, liver, stomach, and spleen. The organs are either located randomly with respect to the left-right (L-R) axis or to one another. This genetic disorder is characterized by multiple congenital malformations and complex cardiovascular malformation that is reported to be the major cause of morbidity and mortality in this population (Table1.1).

Heterotaxy accounts for approximately 3% of all congenital heart defects (CHD) with an approximate prevalence of 1:10,000 live births (Lin et al., 2000), most frequently diagnosed in the newborn mainly due to cyanotic congenital heart disease (Sutherland and Ware, 2009). In individuals with heterotaxy any internal organ that is asymmetrically positioned can be abnormal and the midline defect, which results from disclosure of neural tube, occurs in approximately 40% of patients (Zhu et al., 2006). The wide phenotypic spectrum seen in heterotaxy, in combination with the lack of formal clinical guidelines for the evaluation, has made the identification of patterns of laterality defects more complicated. Thus these patients require an extensive diagnostic evaluation and multiple imaging studies in order to define their anatomy as well as chromosome microarray analysis due to associations with chromosome abnormalities.
Table 1.1: Congenital anomalies associated with heterotaxy (Zhu et al., 2006)

| **Cardiovascular** | Atrioventricular discordance  
|                  | Atrial isomerism  
|                  | Double outlet right ventricle  
|                  | Pulmonary stenosis or atresia  
|                  | Transposition of the great arteries  
|                  | Single ventricle  
|                  | Left ventricular outflow tract obstruction  
|                  | Septal defects  
|                  | Total/partial anomalous venous return  
|                  | Interrupted inferior vena cava  
|                  | Bilateral superior vena cava  
|                  | Conduction system abnormalities  
| **Gastrointestinal** | Extrahepatic biliary atresia  
|                  | Intestinal malrotation  
|                  | Omphalocoele  
|                  | Duodenal atresia/obstruction  
|                  | Tracheoesophageal fistula  
|                  | Annular pancreas  
|                  | Microgastria  
|                  | Midline liver  
|                  | Right-sided stomach  
| **Pulmonary** | Hyparterial or eparterial bronchi  
|                  | Impaired mucociliary clearance in patients with ciliary defect  
| **Central nervous system** | Neural tube defect  
| **Genitourinary/anal** | Imperforate anus  
|                  | Sacral dysplasia  
|                  | Renal dysplasia  
|                  | Horseshoe kidney  
|                  | Fused adrenal glands  
|                  | Bifid or septate uterus and vagina  
| **Musculoskeletal** | Vertebral and rib anomalies  
| **Immune** | Asplenia or polysplenia  
| **Ear, Nose, and Throat** | Cleft palate  

2
1.2-PIDEMIOLOGY AND INHERITANCE OF HETEROTAXY AND OTHER DISORDERS OF L-R ASYMMETRY

Situs inversus, referring to complete mirror image arrangement of all internal organs, is estimated to occur in 1 in 8,000-25,000 individuals and is mainly due to abnormal cilia function or absent of cilia lining the respiratory tract known as primary ciliary dyskinesia (PCD). PCD is also associated with a wide variety of human diseases such as bronchitis, infertility, hydrocephalus, anosmia, and retinitis pigmentosa (Afzelius et al., 2001; Pan et al., 2005), and has an autosomal recessive inheritance pattern.

Situs ambiguous, which refers to abnormal arrangement of internal organs with discordance of thoracic and visceral anatomy is mainly characterized by congenital anomalies and is estimated to take place in 1:10,000 live births (Lin et al., 2000). Most cases of situs ambiguous occur sporadically and do not follow obvious Mendelian inheritance patterns, although phenotypic characterization of families suggests both autosomal recessive and dominant patterns with incomplete penetrance due to evidence of familial clustering. Family studies have identified isolated cardiac defects, isolated neural tube defects, or abnormal vasculature in unaffected individuals (Morelli et al., 2001).

Laterality defects, which refer to any deviation from situs solitus including the failure to generate asymmetry, can arise in a single individual (Kosaki and Casey, 1998; Winer-Muram, 1995) but are especially associated with monozygotic twinning. Environmental factors such as exposure to drugs or chemicals such as
nitrous oxide, retinoic acid, phenylephrine, methoxamine, have been reported to associate with L-R phenotypes in animal models (Levin, 2005).

Isolated dextrocardia, which refers to right-sided heart position, showed a strong association with a family history of CHD and extracardiac anomalies. A population based study of cardiovascular malformation indicated that maternal diabetes and first trimester cocaine use are associated with heterotaxy (Kuehl and Loffredo, 2002).

Heterotaxy most often occurs as a sporadic condition. Genetic mechanisms underlying heterotaxy are still not completely known, but the familial studies have suggested a complex inheritance or autosomal dominant and autosomal recessive inheritance with reduced penetrance and variable expressivity. (Vitale et al., 2001; Belmont et al., 2004; Zhu et al., 2006; Wessels et al., 2008).

X-linked inheritance is well documented in heterotaxy and is caused by mutations in ZIC3 (Gebbia et al., 1997). A large number of genetic syndromes and chromosome abnormalities have been associated with heterotaxy, including aneuploidies, complex chromosomal rearrangements, and microdeletions (Bisgrove et al., 2003; Ware and Belmont, 2008; Song et al., 2009). Reported examples are patients with trisomy 13 or trisomy 18. In addition, a number of submicroscopic chromosomal deletions, including 22q11.2 DiGeorge/Velocardiofacial Syndrome (VCFS) syndrome, have been identified in patients with heterotaxy. Bisgrove et al. (2003) reviewed unbalanced translocations, terminal deletions, inversions, and more complex chromosomal rearrangements associated with heterotaxy.
Analysis of genes located within submicroscopic chromosomal imbalances in patients with abnormal L–R asymmetry should be a useful tool in uncovering novel genetic pathways in laterality defects. However, it is not yet clear what percentage of isolated cardiac defects are mechanistically related to early L-R patterning abnormalities. In addition, the true incidence of human L-R patterning defects is not yet known and so further definition of the molecular basis may identify a higher incidence that has been recorded and a better understanding of this genetic disorder.

1.3-L-R AXIS ESTABLISHMENT DURING EMBRYONIC DEVELOPMENT

The congenital disorder in patients with heterotaxy mainly results from failure to establish normal L-R asymmetry during embryonic development, with major morbidity and mortality resulting from complex cardiovascular malformations (Sutherland and Ware, 2009). The L-R asymmetry of the heart is closely connected to its function and alteration in cardiac situs represent a significant source of human heart malformations (Kathiriya and Srivastava, 2000). Since the L-R axis is established during early development, a basic understanding of an embryonic tissues and molecular events involved in L–R axis formation is beneficial for initiating a diagnostic work-up, understanding the genetic basis of disease, and assessing recurrence risk.

The L-R axis is established early in embryogenesis subsequent to the development of anterior-posterior (A-P) and dorsal-ventral (D-V) axes. Recent work
with model organisms has offered a detailed view of the steps taken to establish asymmetry in the vertebrate (Bisgrove and Yost, 2001). In Fig.1.1, five steps in developing L-R asymmetry in the mouse embryo are illustrated. The early stage of L-R patterning occurs before cilia are established at the node, a transient embryonic organizer. This involves processes such as planar cell polarity in which asymmetric gradients are established within a cell, across a sheet of cells and ultimately throughout the embryo (Aw and Levin, 2009). The signaling pathways and the molecular mechanisms of early stage of L-R patterning during embryogenesis are not yet well defined and may vary by model organism (Sutherland and Ware, 2009).

![Figure 1.1](image)

**Figure 1.1:** Overview of L-R asymmetry in mouse embryo (Sutherland and Ware, 2009). Evidence shows intercellular asymmetry involving mechanism such as Planar cell polarity (PCP) take place before the establishment of the node or cilia. During the second stage, the node and functioning motile cilia form. Centrally located monocilia are motile and peripheral monocilia lack L–R dynein and are immotile. In the next stage, the movement of cilia at the node creates a leftward flow of morphogens. Nodal expression is initially bilateral, but becomes asymmetrically expressed on the left, providing molecular evidence of L–R asymmetry. During the stage four, Nodal signal is propagated from the node to the lateral plate mesoderm (LPM) where asymmetric gene expression is established. In stage five, asymmetric signaling is propagated from the LPM to organ primordia in order for proper morphogenesis to occur.
During the second stage of mouse embryo development, the node and functioning motile and sensory cilia form. The genetic control of node morphogenesis is unknown but it has been proven that Notch signaling, which allows a cell-cell communication, is necessary for both proper node structure as well as later asymmetric nodal expression (Krebs et al., 2003; Przemeck et al., 2003; Takeuchi et al., 2007). In addition, at this stage the monocilia located at center are motile compare to immotile peripheral monocilia (McGrath et al., 2003). This characteristic allows the movement of cilia at the node, creating a leftward flow of morphogens, which form the clockwise rotational motion of the cilia and creating a unidirectional flow in stage 3, which is essential for proper L-R asymmetry (Sutherland and Ware, 2009).

In the next stage, as results of leftward flow of cilia, fibroblast growth factor (FGF) signaling triggers the releases of particles carrying the morphogens Sonic hedgehog and retinoic acid, which are pushed to the left of the node and trigger a cascade of gene expression (Okada et al., 2005; Hirokawa et al., 2006). In addition, the unidirectional flow of extracellular fluid bends the peripheral immotile cilia surrounding the node, resulting in an asymmetric cytoplasmic calcium flux (McGrath et al., 2003). These two pathways effect Nodal expression which initially is bilateral, but becomes asymmetrically expressed on the left, providing the first molecular evidence of L-R asymmetry which is critical for subsequent left-sided gene expression in the lateral plate mesoderm (LPM) (Sutherland and Ware, 2009).
In stage four, Nodal signal transduction pathway expressed in the left side of the node and the signal is propagated to the LPM where asymmetric gene expression is established. At the presence of an EGF-CFC cofactor, Nodal signals through type I and type II receptors and activates two antagonists Lefty-1 (LEFTYA in human) and Lefty-2 (LEFTYB in human) which are expressed in the midline and in the LPM, respectively. This activation limits Nodal expression and subsequently inhibits the transfer of left-sided gene expression across the midline of the embryo. Pitx2, a homeobox transcription factor, is also activated by Nodal and is asymmetrically expressed in the LPM as well as in various organs. This molecular asymmetry is much conserved across vertebrates and the majority of the known human heterotaxy mutations have been discovered in this pathway. However, mutations within this pathway cause heterotaxy in a minority of cases, indicating that novel genetic etiologies remain to be determined (Sutherland and Ware, 2009).

In the final stage, asymmetric signaling is propagated from the LPM to organ primordia in order for proper morphogenesis to occur. The asymmetric Pitx2 expression persists after the disappearance of the Nodal signal, and involves in organogenesis in the heart, gut, and lungs, demonstrating asymmetric expression in developing organ primordia. Information about cardiac specific targets during looping morphogenesis is currently lacking and needs further investigation.

Study of L-R asymmetry using model organism has shown that heterotaxy may result from defects in any of these five steps. Isolated dextrocardia or CHD related to abnormal laterality can also result from abnormalities at various stages of
L-R patterning. However, a detailed genetic mechanism of the pathway is still remains to be determined.

\[1.4\text{-Clinical and prenatal evaluation of Heterotaxy}\]

\[1.4.1\text{-The clinical evaluation of heterotaxy}\]

Although heterotaxy shows broad phenotypic variability, the approach to studying this population is relatively straightforward. The first and most important step during clinical evaluation of laterality defects is to identify the full phenotypic spectrum of abnormalities in the proband and to determine whether it is a case of situs inversus or situs ambiguous. This information is essential for patient management as well as providing information that is critical for identification of the possible underlying genetic etiology.

Patients with situs inversus should have evaluation of ciliary function. In addition the signs of Bardet-Biedl syndrome (BBS), including obesity, retinitis pigmentosa, and mental retardation, should be evaluated. Although situs inversus is infrequently reported in BBS, it occurs at a significantly higher rate than in the general population (Ansley et al., 2003). Mutations in \textit{BBS2} and \textit{BBS8} have been reported in association with situs inversus. Five out of eight known \textit{BBS} genes encode basal body or cilia proteins, implying a potential for laterality defects (Beales, 2005). Infantile nephronophthisis is another genetic disorder that is associated with situs inversus. This is an autosomal recessive cystic kidney disease which is caused by mutations in \textit{INVERSIN} (Otto et al., 2003). Lacking evidence of
cystic kidneys or stigmata of BBS, a patient with situs inversus is classified as having PCD, previously known as immotile cilia syndrome, and therefore and should be followed by a pulmonologist or allergist/immunologist as needed (Zhu et al., 2006).

Patients with situs ambiguous require an extensive diagnostic evaluation directed toward defining the anatomy and preventing potential complications. The evaluations tailored based on data collected from performing an echocardiogram and/or cardiac MRI, abdominal and renal ultrasound, and vertebral X-rays. In addition, the spleen should be evaluated by ultrasound and a peripheral blood smear to detect the presence or absence of Howell-Jolly bodies, red blood cell inclusions indicating abnormal spleen function.

A three-generation pedigree is an essential component of the evaluation. For situs ambiguous, any family history of congenital anomaly is potentially contributory. In addition, mouse models suggest that increased pregnancy loss may be associated with mutations in genes responsible for L-R patterning (Zhu et al., 2006). In addition, there are large numbers of other syndromes that are associated with laterality defects that can be used for clinical evaluation (Aylsworth, 2001). Among aneuploidies, both trisomy 13 and trisomy 18 can cause congenital anomalies overlapping situs ambiguous that can potentially complicate prenatal counseling if amniocentesis is not performed (Ware and Belmont, 2004). Microdeletion 22q11.2 can be detected by karyotype which is associated with laterality defects (Aylsworth, 2001). It is likely that a number of complex malformation syndromes result from early developmental patterning defects in which laterality can be disrupted.
1.4.2-Prenatal evaluation

The frequency of identified heterotaxy cases on prenatal ultrasound has increased as the result of advances in imaging technology (Berg et al., 2003). For example, fetal echocardiography has provided a tool for detailed study of the structure of an unborn baby’s heart. Early fetal heart block is commonly associated with left isomerism and early detection of the disorder is critical for clinical management (Jaeggi et al., 2005). In the absence of a syndromic diagnosis, the prognosis of the fetus is largely based on the cardiac anatomy and/or the presence of biliary atresia. Biliary atresia is a disorder of infants in which there is progressive obliteration or discontinuity of the extrahepatic biliary system, resulting in obstruction of bile flow, requiring liver transplantation within the first year of life (Leyva-Vega et al., 2010). Biliary cystic malformations can be identified prenatally and, although not specific for biliary atresia, are highly suspicious when identified in conjunction with other laterality defects. Since the inheritance patterns of heterotaxy is very complicated and varies among individuals, genetic counseling in prenatal cases is complex. In addition to discussion of the anatomic defects and their prognosis and management, counseling sessions should also include discussion of both syndromic and nonsyndromic possibilities and an explanation of inheritance patterns. However, to gain a better understanding of these disorders, analysis of genes involve in L-R asymmetry would be a powerful tool in uncovering novel genes and pathways in laterality defects.
1.5-**Genetic risk factors and molecular diagnostics**

Model organism studies have shown that functions of more than eighty genes are required for normal asymmetric L-R organ development. While some of these genes probably have a conserved function in humans and any mutation in them are lethal, mutations in relatively few genes have been identified in patients with heterotaxy. These include \textit{ZIC3}, \textit{LEFTYA}, \textit{CRYPTIC} and \textit{ACVR2B}, and single cases of mutations in \textit{NKX2.5} and \textit{CRELD1} have been reported in patients with dextrocardia (Watanabe et al., 2002; Robinson et al., 2003). The contribution of mutations in these genes to heterotaxy or L-R patterning defects in the heart remains to be characterized further in larger sample sets (Zhu et al., 2006). In all these mutation studies, conventional methods of assessing the role of particular rare variants as mutations have been used. This usually includes analysis of controls for the particular variant. However, there is very little power to repeatedly detect rare variants, even in very large control sets.

1.5.1-**Zinc finger protein ZIC3**

The zinc finger domains (ZFDs) of the Zinc family proteins have been strongly conserved over the evolution of a broad range of eumetazoan animals (Aruga et al., 2006) suggests they provide the structural basis for the essential roles of Zic family protein in animal development (Aruga, 2004; Herman and El-Hodiri, 2002). In humans there are five members of the ZIC family that are involved in
congenital anomalies (Grinberg and Millen, 2005). One of them, \textit{ZIC3}, was the first gene identified to have a casual role in defects in human laterality (Ferrero et al., 1997; Gebbia et al., 1997). Mutations in \textit{ZIC3}, which is on the X chromosome, can cause the X-linked form of heterotaxy, HTX-1 (OMIM#306955).

\textit{ZIC3} was first identified as a gene preferentially expressed in mouse cerebellum (Aruga et al., 1996) and has the ability to bind DNA and activate transcription (Mizugishi et al., 2001). Zic3-deficient mice develop a broad range of abnormalities in addition to heterotaxy such as neural tube defects, skeletal patterning defects, cerebellar hypoplasia and abnormal behavior (Carrel et al., 2000; Klootwijk et al., 2000; Purandare et al., 2002; Aruga et al., 2004).

\textit{ZIC3} protein is made of five tandemly repeated C2H2 zinc finger transcription factors that are involved in early patterning of the vertebrate embryo. In human, missense mutations that result in changes in cysteine 253 (C253S) or histidine 286 (H286R) are found in heterotaxy patients and cause extracellular localization of the mutant \textit{ZIC3} protein (Ware et al., 2004). Another mutation that alters a tryptophan residue (W255G) is associated with transposition of the great artery (TGA), a CHD that might be an expression of a L-R laterality disturbance (Chhin et al., 2007). Loss of function of this gene has also been reported to associate with other abnormalities such as double outlet right ventricle, and ventricular inversion (Sutherland and Ware, 2009).

Approximately 1\% of sporadic heterotaxy cases (male and female) are caused by mutation in \textit{ZIC3}. In addition, affected females have been described with point
mutations or with chromosomal translocations at this gene (Megarbane et al., 2000; Ware et al., 2004; Fritz et al., 2005; Tzschach et al., 2006; Chhin et al., 2007).

Carrier females who are heterozygous for ZIC3 mutation are clinically unaffected although a subset has situs inversus (Casey et al., 1993; Gebbia et al., 1997). Deletion of the ZIC3 locus has been associated with situs ambiguous, suggesting the cause of pathogenesis in the patients with the point mutations (Ferrero et al., 1997).

These findings have suggested that isolated CHD may be caused by genes involved in L-R axis development and that there may be variable expression of mutant alleles within and between families. It is currently unclear to what degree ZIC3 mutations contribute to sporadic heterotaxy, and this information is necessary to provide accurate counseling information to families. Mice models suggest that ZIC3 acts upstream of Nodal signaling at the node, however its detailed developmental function is not yet clear (Purandare et al., 2002; Ware et al., 2006).

1.5.2- NODAL, LEFTYA, CRYPTIC, and ACVR2B

NODAL is a member of the transcription growth faction (TGF)-β family and is involved in cell differentiation. The pattern of NODAL expression and its developmental function has largely conserved among vertebrates. In the mouse, it is known to have an essential role in early embryonic development including mesoderm and endoderm formation and L-R axis patterning. During gastrulation stage of development, NODAL is expressed in the epiblast and visceral endoderm, and it
induces its co-receptor Cripto, which controls A-P patterning. Lack of NODAL expression in mice causes the absence of primitive streak and loss of mesoderm formation (Conlon et al., 1994; Lowe et al., 1996; Zhou et al., 1993). The functions of NODAL have been examined in other species, and in all of them NODAL orthologs are expressed asymmetrically in the left side of the node as well as in left lateral plate mesoderm (Schier and Shen, 2000; Whitman, 2001). This data shows that the Nodal signaling cascade within the lateral plate mesoderm is required for L-R patterning in vertebrates (Mercola and Levin, 2001; Hamada et al., 2002; Schier, 2003), and any disturbance in expression of NODAL can lead to polarity reversal of visceral organs and heart looping (Harvery, 1998). Mutations in NODAL have been identified in patients with heterotaxy following an autosomal dominant inheritance pattern (Kosaki et al., 1999; Bamford et al., 2000; Goldmuntz et al., 2002; Selamet Tierney et al., 2007; Roessler et al., 2008; Mohapatra et al., 2009).

Nodal signaling uses an Activin/TGF-β-like pathway mediated by several Activin-like receptors (ALKs). NODAL signals via ALK4 and AKL7 in association with either ActRIIA or ActRIIB (Whitman 2001). The activated receptor complex phosphorylates an intracellular receptor-regulator known as Smads. This phosphorylated complex binds to Smad4 and that results in its translocation to the nucleus (Attisano and Wrana, 2002). This complex then can either bind DNA directly or can interact with other DNA binding proteins such as transcription co-activator FoxH1 to regulate the promoters of various genes. In addition, FoxH1 regulates Nodal signaling pathway by binding to the NODAL and Lefty2 asymmetric
enhancer element (Saijoh et al., 2000). Mutations in several components of the Nodal signaling pathway in animal models has exhibit defects in L-R axis development (Pogoda et al., 2000; Yamamoto et al., 2001; Yan et al., 2002). Mutation in components of the Nodal signaling pathway, such as genes encoding the ligand (NODAL), ligand co-receptor (CFC-1), receptor (ACVRIIB), FOXH1, and midline inhibitor (LEFTYA) within the Nodal signal transduction pathway, have been identified in human heterotaxy (Kosaki et al., 1998; Bamford et al., 2000; Goldmuntz et al., 2002; Selamet Tierney et al., 2007; Roessler et al., 2008; Mohapatra et al., 2009). Biochemical studies indicate that Cripto (TDGF1) and Cryptic (CFC1) in mouse and human form complexes with NODAL, ALK4/ALK7 and ActRIIB and act as a co-receptor for NODAL at the cell surface (Bianco et al., 2002; Yan et al., 2000).

Study on NODAL variants in 269 patients with heterotaxy or isolated cardiovascular observed missense mutations in 14 unrelated patients (Mohapatra et al., 2009). Functional analysis of the mutant proteins showed a decrease in Nodal signaling. Similarly, functional analysis of mutations in Cryptic (CFC1), a gene encoding an epidermal growth factor family protein, CFC1, which functions as a NODAL co-receptor, revealed abnormal cellular localization of the mutant protein (Bamford et al., 2000). ACVRIIB mutations were identified in 3 of 126 patients with left–right anomalies and mutations in LEFTYA were discovered in 2 patients (Kosaki et al., 1998). The study showed that each patient inherited the mutant allele from an
unaffected carrier parent, indicating autosomal recessive inheritance pattern or incomplete penetrance.

1.5.3-Other risk factors

With over hundred candidate genes identified through mouse models of L–R patterning defects, it is likely that significant genetic heterogeneity will be found in human heterotaxy. To date, the majority of genes identified have either not yet been tested in larger heterotaxy populations or have been found to have mutations at a low frequency (Watanabe et al., 2002; Robinson et al., 2003). For example, positional cloning of a region involved in a reciprocal translocation in one patient identified SESN1, a gene involved in L-R patterning. Functional analysis of this protein revealed that it plays a role in activating Nodal signaling. However, the prevalence of disease causing mutations in this gene is less than 1% based on an initial screen of a heterotaxy cohort (Peeters et al., 2003, Peeters et al., 2006). Efforts are ongoing to identify novel genes that cause or contribute to the heterotaxy phenotype. One of the candidate genes that require a large population study in patients with heterotaxy is CRELD1. Missense mutations in CRELD1 are associated with atrioventricular septal defects (AVSD), including AVSD in patients with heterotaxy (Robinson et al., 2003).
1.6-**CRELD1 as a Candidate Gene**

CRELD1 is the member of a highly conserved family of proteins known as CRELD (Cysteine Rich with EGF-Like Domains) (Rupp et al., 2002), that are members of the TGF-superfamily, which contains a diverse array of multifunctional cytokines. This group has one other homologous member, CRELD2. The two members of this family are nearly identical except that CRELD1 is a cell surface molecule, whereas CRELD2 is a secreted protein that is possibly a regulator of CRELD1 function. These cell-surface/extracellular proteins are characterized by tandemly repeated epidermal growth factor-like (EGF) domains and a unique motif called the CRELD domain.

**CRELD1** is located on chromosome 3p25 (Rupp et al., 2002) and is the first recognized risk factor for isolated AVSD (Robinson et al., 2003), a common congenital heart malformation affecting the atrioventricular valves and septa that results in a mixing of oxygenated and non-oxygenated blood. In addition, **CRELD1** is becoming increasingly recognized as an important factor in cancer biology with numerous proposed biochemical interactions.

**CRELD1** missense mutations occur in almost 8% of individuals with AVSD (Robinson et al., 2003), and 5% of Down syndrome-associated AVSD cases (Maslen et al., 2006). The highly specific genetic association between **CRELD1**-missense mutations and AVSD suggests that mutations in **CRELD1** significantly increase the risk of developing an AVSD. High level of incidence of AVSD has been reported in heterotaxy patients, presumably due to physical disruption of septation from
laterality defects. Missense mutations in \textit{CRELD1} suggest that defects in \textit{CRELD1} may increase the likelihood of AVSD in heterotaxy (Robinson et al., 2003).

However, a recent study on a \textit{CRELD1} knocked out (\textit{Creld1}^{(-/-)}) mice model indicates that \textit{CRELD1} is not associated with laterality defects in mice embryos (Redig et al., submitted).

Fig.1.2 compares \textit{Creld1}^{(-/-)} mice embryos with a \textit{Creld1}^{(+/-)} and wild type \textit{Creld1}^{(+/-)} embryos. As can be seen, \textit{Creld1}^{(-/-)} embryos exhibited underdeveloped organs such as telencephalons and heart. However, the positions of organs is the same in \textit{Creld1}^{(-/-)} embryos relative to wild-type \textit{Creld1}^{(+/-)} with no sign of laterality defects. This indicates that \textit{CRELD1} does not cause laterality defects (Redig et al., submitted), but it doesn’t rule out the possible role of \textit{CRELD1} as a risk factor for developing AVSD in a heterotaxy population.

The basic biological function and definition of the \textit{CRELD1} biochemical pathway is under investigation. Multiple alternate splice variants of \textit{CRELD1} suggest that there are isoforms with diverse functions. \textit{CRELD1} can bind to RTN3, an apoptosis-inducing protein responsive to endoplasmic reticulum stress, and controls apoptosis activity of RTN3 (Xiang and Zhao, 2009). In addition, a recent study on cell migration during development suggests the important role of \textit{CRELD1} in polarized cell migration (Nystul et al., 2009), a process regulated by Notch signaling which is necessary for proper node structure as well as asymmetrical nodal expression.
Figure 1.2: Mice embryos at the developmental stage (Redig et al., submitted). The two picture on far left (A, A’) are showing the wild-type embryo, two in the middle (B, B’) are heterozygous for Creld1\(^{+/+}\) and the two other on the far right (C, C’) are showing the knocked out mice Creld1\(^{-/-}\) embryos. There is a broad abnormalities in Creld1\(^{-/-}\) embryos especially in the head, which exhibited underdeveloped telencephalons, T and underdeveloped olfactory placodes, OP. The position of Limb buds (LB), olfactory placode (OP), branchial arch (BA), telencephalon (T), heart (H) are indicating the absence of laterality defects in Creld1\(^{-/-}\) embryos.
1.7-Hypothesis

Despite all of the indications that CRELD1 plays a role in human early development, no study has been done in large sample of patients with heterotaxy to determine if mutations in CRELD1 are a risk factor increasing the likelihood of AVSD in this population. To address this, we examined whether there is a significant difference between the variables in this population. Our hypothesis and alternative hypothesis are:

- **Hypothesis:** there are missense mutations in CRELD1 which increase the likelihood of AVSD or other CHD in heterotaxy patients.
- **Alternative Hypothesis:** there is no novel mutation in CRELD1 effecting susceptibility for CHD in this population.
CHAPTER II: METHOD AND MATERIALS

2.1-Clinical Evaluation and Sampling

The study sample included 126 individuals with a diagnosis of heterotaxy syndrome with or without heart defects, identified by the Baylor College of Medicine, Houston, Texas and provided in collaboration with Dr. John Belmont. The study population ranged broadly in age and sex.

A biological sample for genomic DNA isolation was obtained from each individual, from blood or by buccal samples collected by mouthwash, by the Belmont group using standard technology. The normal control population DNA panels from Caucasian and Hispanic populations were obtained from the Coriell Repository (Camden, NJ).

2.2-Design of Oligonucleotide Primers

The coding and known regulatory regions of CRELD1 were screened for point mutations by standard DNA sequencing of PCR amplified genomic DNA. PCR amplification was done using previously designed primers and conditions (Robinson et al., 2003). The primer sequences and amplification profiles are listed in table 2.1. Primers were used to amplify ten exons and two promoter regions as well as at least 50 bp into introns from each junction in a total of seven amplicons.
2.3-PCR Amplification

For each patient genomic DNA sample, seven PCR reactions were performed using the primers listed in table 2.1. PCR reactions for regions including exon 3, 4, 10, and promoter region 2 were performed in a 25 µl reaction volume containing 100 ng of genomic DNA, 25 pmol of each primer, and 12.5 µl of Invitrogen AccuPrime™ PCR SuperMixII.

For the rest of the exons and promoter region 1, PCR reactions were performed in a 25 µl reaction volume containing 100 ng of genomic DNA, 25 pmol of each primers, 5 µl of Herculase II buffer (5x), 2 µl of 10mm dNTPs, 1.25 µl of DMSO, 0.25 µl of Herculase II Polymerase (Stratagene; La Jolla, CA, USA). PCR cycling was carried out on a Veriti® Thermal Cycler (Applied biosystems, CA, USA) using a denaturation cycle at 95 ºC for 2 min, followed by 35 cycles of denaturation at 95ºC for 15 sec and annealing (Table 2.1) 15 sec, and extension step at 72ºC for 30 sec and a final extension step at 72ºC for 7 min. The products were visualized by standard ethidium bromide-agarose gel electrophoresis.
**Table 2.1:** Oligonucleotide primers for *CRELD1* amplification by PCR.

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Primer pair</th>
<th>Annealing Temperature (°C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
</table>
| *CRELD1* Promoter Region 1 | Pro 1-F 5’ CTG CTT CGA CAG GCT 3’  
Pro 1-R 5’ ATC CGG ATA TGA AGG 3’ | 62                          | 685                |
| *CRELD1* Promoter Region 2 | Pro 2-F 5’ CGG TCG CTT CTT CCT TCT C  
Pro 2-R 5’ AGA AGA CTG GGG AGG TGG AG 3’ | 63                          | 665                |
| *CRELD1* Exons 1-2     | E1-F 5’ AGC CTC TCC ACG CCC TCT A 3’  
E2-R 5’ CCT AGC CCT ACA CCA GCA GAG 3’ | 62                          | 600                |
| *CRELD1* Exons 3-4     | E3-F 5’ GAG ACT TGA GGA GGG TGG TG 3’  
E4-R 5’ AAG CCT TTC CCC ACT GAT TT 3’ | 58                          | 800                |
| *CRELD1* Exons 5-6     | E5-F 5’ TGT ATA GAT GAC CTC ACC TGG TTT 3’  
E6-R 5’ CTG ACC ATC TTC CCA GAC CT 3’ | 58                          | 400                |
| *CRELD1* Exons 7-8-9   | E7-F 5’ CTC TGG CTT CAG CTT CCC TA 3’  
E9-R 5’ GCC TGC CTT CTC TTT GAA TG 3’ | 63                          | 800                |
| *CRELD1* Exon 10       | E10-F 5’ CCA GGA ACA GGG ATA CGA GTG 3’  
E10-R 5’ GGT GCT TAC CCC ACT CTC AAA 3’ | 58                          | 400                |

**2.4-PURIFICATION OF PCR PRODUCTS**

Template quantity was adjusted based on the PCR product size (Table 2.2). 5 µl of PCR product was purified using 2 µl of ExoSAP-IT® (United States Biochemical, Ohio, USA). The samples were incubated at 37°C for 15 min to degrade remaining primers and nucleotides followed by incubating at 80°C for 15 min to inactivate ExoSAP-IT.
Table 2.2. Template quantities for 1X cycle-sequencing run

<table>
<thead>
<tr>
<th>Template</th>
<th>Template quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR product:</td>
<td></td>
</tr>
<tr>
<td>100-200 bp</td>
<td>1-3 ng</td>
</tr>
<tr>
<td>200-500 bp</td>
<td>3-10 ng</td>
</tr>
<tr>
<td><strong>500-1000 bp</strong></td>
<td><strong>5-20 ng</strong></td>
</tr>
</tbody>
</table>

2.5-SEQUENCE ANALYSIS

The templates were sequenced in both directions using the sequencing primers, listed in Table 2.3, by the OHSU General Clinical Research Center DNA Sequencing Facility. The sequencing electropherograms were compared with gene sequences from GenBank accession number NM_001077415.1 using Mutation Surveyor™ DNA Analysis software (SoftGenetics LLC, Inc).

Table 2.3: Oligonucleotide primers for CRELD1 exon sequencing

<table>
<thead>
<tr>
<th>Region</th>
<th>Primer pair</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promoter Region 1</td>
<td>Pro 1-F-Seq 5’ CTG CTT CGA CAG GCT 3’</td>
</tr>
<tr>
<td></td>
<td>Pro 1-R-Seq 5’ ATC CGG ATA TGA AGG 3’</td>
</tr>
<tr>
<td>Promoter Region 1</td>
<td>Pro 2-F-Seq 5’ CGG TCG CTT CTT CCT TCT C</td>
</tr>
<tr>
<td></td>
<td>Pro 2-R-Seq 5’ AGA AGA CTG GGG AGG TGG AG 3’</td>
</tr>
<tr>
<td>CRELD1 Exons 1-2</td>
<td>E1-F-Seq 5’ CGC CCT CTA TCT GCA GGT CC 3’</td>
</tr>
<tr>
<td></td>
<td>E2-R-Seq 5’ GAG CAG AGA TTT GGC GGG GAG GGG A 3’</td>
</tr>
<tr>
<td>CRELD1 Exons 3-4</td>
<td>E3-F-Seq 5’ GGG TGG TGG GTG TGG GGG GGC 3’</td>
</tr>
<tr>
<td></td>
<td>E4-R-Seq 5’ CCC ATC TCC CCA GCC CTC GC 3’</td>
</tr>
<tr>
<td>CRELD1 Exons 5-6</td>
<td>E5-F-Seq 5’ CAC CTG CTT TGG TGT CTT CC 3’</td>
</tr>
<tr>
<td></td>
<td>E6-R-Seq 5’ TCC CAG ACC TAG CTA GGA CCG CC 3’</td>
</tr>
<tr>
<td>CRELD1 Exons 7-8-9</td>
<td>E7-F-Seq 5’ TTC AGC TCC ACT AAA TAG GGG 3’</td>
</tr>
<tr>
<td></td>
<td>E9-R-Seq 5’ TCT TTG AAT GAG GAC CTC CC 3’</td>
</tr>
<tr>
<td>CRELD1 Exon 10</td>
<td>E10-F-Seq 5’ TAC GAG TGC CAG GCT GCA TC 3’</td>
</tr>
</tbody>
</table>
2.6-**Allele-Specific PCR (ASP)**

The Single Nucleotide Polymorphism (SNP) database was queried to identify DNA sequence alterations that were commonly accruing SNPs. In order to detect SNPs for missense amino acid changes found at exon 5, amino acid position A206T, first detected on sequencing data, allele-specific PCR analysis was used on the patient sample and control population.

Genomic DNA was amplified by use of primers that match/mismatch one of the alleles at the 3’-end of the reverse primer (5’-CATACCAGATGGCTGGTG-3’ and 5’- CATACCAGATGGCTGGCG-3’, mutant and wild type, respectively). The reverse primers were coupled with upstream (forward) primer (5’-CTGGTTTGGTGTCTTCCC -3’). Characterization of this assay demonstrated that the wild-type exon 5 primer amplified genomic DNA from both the patient and normal controls, whereas the mutant primer was capable of amplifying only genomic DNA from the affected individual in whom the mutation had been characterized.

**Table 2.4:** ASP primer sequence.

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence</th>
<th>PCR condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRELD1-A206T-200-F</td>
<td>CTG GTT TGG TGT CTT CCC</td>
<td>95 ºC x 2’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95 ºC x 15’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>62 ºC x 15’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72 ºC x 15’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72 ºC x 7’</td>
</tr>
<tr>
<td>CRELD1-A206T-2nd-mut-R</td>
<td>CAT ACC AGA TGG CTG GTG</td>
<td>95 ºC x 2’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95 ºC x 15’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>62 ºC x 15’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72 ºC x 15’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72 ºC x 7’</td>
</tr>
<tr>
<td>CRELD1-A206T-2nd-WT-R</td>
<td>CAT ACC AGA TGG CTG GCG</td>
<td>95 ºC x 2’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95 ºC x 15’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60 ºC x 15’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72 ºC x 15’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72 ºC x 7’</td>
</tr>
<tr>
<td>CRELD1-T265A-2nd-mut-R</td>
<td>CTC ATA GGA GCC CTC AGC</td>
<td>95 ºC x 2’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95 ºC x 15’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60 ºC x 15’</td>
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<tr>
<td></td>
<td></td>
<td>72 ºC x 15’</td>
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<tr>
<td></td>
<td></td>
<td>72 ºC x 7’</td>
</tr>
<tr>
<td>CRELD1-T265A-2nd-WT-R</td>
<td>CTC ATA GGA GCC CTC AGT</td>
<td>95 ºC x 2’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95 ºC x 15’</td>
</tr>
<tr>
<td></td>
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<td>60 ºC x 15’</td>
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<td></td>
<td></td>
<td>72 ºC x 15’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72 ºC x 7’</td>
</tr>
<tr>
<td>CRELD1-T265A-200-F</td>
<td>GGT TGG CAA ATG TGG TCT C</td>
<td>95 ºC x 2’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95 ºC x 15’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60 ºC x 15’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72 ºC x 15’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72 ºC x 7’</td>
</tr>
</tbody>
</table>
The DNA samples were amplified by PCR using 100 ng of genomic DNA in a 25 µl reaction containing 0.2 µl of 25 mM of each primer, 5 µl of Herculase II buffer (5x), 2 µl of 10mm dNTPs, 1.25 µl of DMSO, 0.25 µl of Herculase II Polymerase (Stratagene; La Jolla, CA, USA), and PCR cycling was carried out on a Veriti® Thermal Cycler (Applied biosystems, Ca, USA). Condition for PCR were 2 min of initial activation step at 95 ºC followed by 40 cycles of denaturation at 95 ºC for 15 sec and annealing at 62 ºC for 15 sec, and extension at 72 ºC for 30 sec and a final extension step at 72 ºC for 7 min. The products were visualized by standard ethidium bromide-agarose gel electrophoresis. In all cases the primers gave a single band of the expected size, 200 bp.

2.7-POLYMORPHISM PHENOTYPING (POLYPHEN-2)

PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2) was used to predict possible impact of an amino acid substitution on the structure and function of CRELD1. Polyphen-2 is a bioinformatics tool which uses multiple algorithms to predict the impact of missense mutations on human protein structure. This tool allows the analysis of all human non-synonymous SNPs publicly available via SNP database.
CHAPTER III: RESULTS

Out of 126 patients with heterotaxy, approximately 66% of them were also diagnosed with different type of heart defects (Table 3.1). DNA sequence analysis of CRELD1 in this cohort of 126 study subjects identified three different missense variants (A206T, R329C and T265A) in three unrelated individuals, who had no other mutations or rare variants identified in their genomes. A two base pair GA deletion was found in non-coding sequence of ten other patients in intron position 9959990/1. Four previously characterized common polymorphic variants (rs279552, rs57022843, rs9853613, rs2302786) were also observed in this population but not investigated further. In addition, three other synonymous variants (L304L, P315P and Q368Q) previously seen by our laboratory in other studies were detected (Table 3.2), but were not further characterized as they appeared to have no functional significance.
Table 3.1: The frequency of different abnormalities found in heterotaxy population of study. Other heart defects were including right sided cardiac apex, mesocardia, hypoplastic left heart syndrome, Left Atrial isomerism, double aortic Arch, L-loop Patent ductus arteriosus, double outlet right ventricle, hypoplastic left ventricle, and interrupted inferior vena cava

<table>
<thead>
<tr>
<th>Abnormality</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASD, VSD, AVSD with or without other heart defects</td>
<td>8</td>
</tr>
<tr>
<td>D/L-loop TGA</td>
<td>40</td>
</tr>
<tr>
<td>D/L-loop TGA with CoA</td>
<td>8</td>
</tr>
<tr>
<td>D/L-loop TGA and other heart defects</td>
<td>9</td>
</tr>
<tr>
<td>Situs inversus with or without dextrocardia</td>
<td>4</td>
</tr>
<tr>
<td>Dextrocardia with other heart defects</td>
<td>7</td>
</tr>
<tr>
<td>Heterotaxy only</td>
<td>38</td>
</tr>
<tr>
<td>MGA and other heart defects</td>
<td>4</td>
</tr>
<tr>
<td>Other types of heart defects</td>
<td>8</td>
</tr>
</tbody>
</table>

Key: ASD, atrial septal defects; VSD, ventral septal defects; AVSD, Atrioventricular Septal Defect; TGA, transposition of great arteries; CoA, coarctation of the aorta; MGA, malposition of great arteries

3.1-PATIENT1-A206T MUTATION

Patient 1 was diagnosed with AVSD and dextro-Transposition of the Great Arteries (d-TGA), referred as a complete transposition of the great arteries (Fig.3.4, B). Analysis of the subject’s DNA revealed a heterozygous missense mutation. The single-base $G \rightarrow A$ substitution, at exon position G9957689GA, and cDNA position 615 (c.615G>A), is located in exon 5 and results in a substitution of threonine for
alanine at amino acid position A206T (Fig.3.2). The amino acid is located in the CRELD domain of CRELD1 (Fig.3.3) with extracellular function. The mutation changes the amino acid located at the sequon position of one of the two N-linked glycosylation sites of CRELD1. Glycosylation is a common post-translational modification for protein molecules involved in cell membrane formation. N-linked glycosylation requires the consensus sequence Asn-X-Ser/Thr which X can be any amino acid but proline. The N-linked glycosylation begins with the addition of a 14-sugar precursor to an asparagine amino acid located at the sequon, and it allows the protein to fold correctly followed by further modification in the endoplasmic reticulum (ER) and Golgi. The first N-linked glycosylation site in CRELD1 is located at amino acid position 79 and the second one is at 205 (Fig.3.1). The mutation at this region is possibly affecting the efficiency of glycosylation.

<table>
<thead>
<tr>
<th></th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAPWPPKGLV PAMLWGLSLF LNLPGPIWLQ PSPPPQSSPP PQPHPCHTCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLVDSFNKGL ERTIRDNFGG GNTAWEE VKNL SKYKDSETRL VEVLEGVCSK</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>SDFECHRLLE SLEE KLVESWW FHKQQEA PD L FQW LCSDSLK LCCPAGTFGP</td>
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<td></td>
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<tr>
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<tr>
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<tr>
<td>TEGANGDQ FCSVTEGSYE CRDCAKACL GCMAGPGCRK KCSPGYQQVG</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>SKCLDVDECE TEVCPEGKQ CENTEGGYRC ICAEGYKQME GICVKEQIE</td>
<td></td>
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<td></td>
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<tr>
<td>SAGFFSEMTE DELVVLQQMF FGlIICALAT LAAGDLVFT AIFIGAVAAM</td>
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</tr>
<tr>
<td>TGYWLRSERSD RVLEGFIKGR</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**Figure 3.1:** CRELD1 protein and the relative position of amino acids. Two N-linked glycosylation sites are shown with the box. The amino acid change is labeled with red color.
As seen in Fig. 3.2 B, the region is conserved within human and bovine but different in mouse. Based on PolyPhen2 analysis this mutation is suggested to have a benign effect on CRELD1 protein structure. The affected subject is of Hispanic descent. The base substitution was not detected in 93 chromosomes from a Hispanic control population indicating that there is \( \leq 5\% \) chance the mutation is a polymorphism in that population with 95\% confidence (Collins and Schwartz 2002) suggesting the variation is unlikely to be a polymorphism.

Figure 3.2: **A.** Sequence analysis identifying missense mutations in *CRELD1*. The arrow on the electropherogram indicates the variant nucleotide, with the wild-type sequence shown below. The altered nucleotide is shown in green. The single-letter amino acid translation is under the first base of each codon. The altered residue is in red. Sequences from the complementary stand showed the same heterozygous change. **B.** Alignment part of the sequence of CRELD domain of human with bovine and mouse.
3.2-PATIENT2-T265A MUTATION

The second alteration was found in a South Asian Indian patient with TGA, coarctation of aorta (CoA), AVSD, and double inlet left ventricle (DILV). The single base substitution of A to G at cDNA position 792 (c. A792G), was identified at exon 7 of DNA patient. This results in a substitution of alanine for threonine at amino acid position 265, located in the highly conserved region of the CRELD domain (Fig.3.5,
A and B). The base substitution was not detected in 102 race-relevant control chromosomes (personal communication, Dr. Subrata K. Dey, West Bengal University of Technology, India). This data indicate that there is less than 5% chance that the mutation is a polymorphism in that population with 95% confidence (Collins and Schwartz, 2002) suggesting the variation is unlikely to be a polymorphism. Polyphen-2 analysis of mutation indicates that the mutation has a structural damaging effect on CRELD1 protein.

**Figure 3.4:** Illustration of different heart defects. A. is showing AVSD also known as canal defect which is a defect involves holes between the two ventricles and atriums. B is showing the TGA where the positions of aorta and pulmonary artery have been reversed. The coarctation of aorta is illustrated in C. D is comparing the normal heart (on the left) with DILV (on the right) where the position of great vessels and ventricles are reversed and both atriums are connected to the left ventricle.
Figure 3.5: A. Sequence analysis data, identifying the second missense mutation in *CRELD1*. The arrow on the electropherogram indicates the variant nucleotide, with the wild-type sequence shown below. The altered nucleotide is shown in green. The single-letter amino acid translation is under the first base of each codon. The altered residue is in red. Sequences from the complementary strand showed the same heterozygous change. B. Alignment of the sequence for the topological domain from human, bovine, and mouse *CRELD1* gene.
**3.3-Patient 3-R329C Mutation**

The third alteration was found in a Hispanic patient with a situs inversus, AVSD, and asplenia, a congenital absence of the spleen which is usually accompanied by complex cardiac malformations, malposition and maldevelopment of the abdominal organs, and abnormal location of the lungs (OMIM# 208530). The patient had a family history of two female siblings who died in utero. The first sibling was diagnosed with pulmonary artery absence (PA hypoplasia), dysplastic tricuspid valve, hypoplastic thymus, asplenia; the second sibling died from cystic hygroma, and liver calcification. No samples were available from the siblings for farther study.

The mutation found in this patient results in a substitution of cysteine for arginine at amino acid 329 (R329C) in exon 9 which is located in a highly conserved region among species (Fig. 3.6, A and B). This amino acid is located in the second calcium-binding EGF (cb-EGF) region, leading to change in $\beta$-sheet folding in the secondary structure of CRELD1 (Robinson et al., 2003). This is a recurrent mutation that has been identified in individuals with non-syndromic AVSD (Robinson et al.), and AVSD with Down’s syndrome (Maslen et al., 2004). The mutation was not detected as a polymorphism in previous studies (Robinson et al., 2003; Kusuma et al., 2011), and has been shown to change the protein conformation (Robinson et al., 2003).
Figure 3.6: A. Sequence analysis data, identifying the third missense mutation in CRELD1. The arrow on the electropherogram indicates the variant nucleotide, with the wild-type sequence shown below. The altered nucleotide is shown in green. The single-letter amino acid translation is under the first base of each codon. The altered residue is in red. Sequences from the complementary stands showed the same heterozygous changes. B. Alignment part of the sequence for the topological domain from human, bovine, and mouse CRELD1 gene.

3.4-Other significant variants in CRELD1

Table 3.2 summarizes the other non-synonymous and synonymous variants found in this heterotaxy population. A non-synonymous variant A to G substituting
valine to methionine at amino acid position M13V is located in exon1. All patients’ DNA samples in this study carried this SNP variant. This SNP was found in database SNP, indicating there is a polymorphism for this variant and suggesting its benign effect on protein structure. PolyPhen-2 analysis also suggested that the mutation is unlikely to change the protein structure or function.

A two base pair GA deletion was found in chromosomes of ten patients, located at a non-coding sequence of CRELD1. The deletion is 52 base pairs upstream of the branch point located in intron eight and probably does not have a significant effect during splicing process. In addition, homozygous substitution of A for G at intron position G9957920A, located at intron 6, was detected in all of the patients’ DNA samples. This SNP is located thirty base pair downstream of intron/exon boundaries, indicating that it is unlikely to affect splicing. No data was found in database for this SNP. Four other known SNPs (rs279552, rs57022843, rs9853613, rs2302786) were also identified for which no significant effect has been reported for them. In addition, three other rare synonymous variants L304L, P315P, and Q368Q were detected. It was observed that all three amino acids coded for CRELD1 are highly conserved among human, mouse, and bovine.

The first synonymous variant was detected as a heterozygous C to T silent mutation at L304L located at the end of the exon 8. Another heterozygous silent mutation was detected in three unrelated individuals. The variant was a substitution of A for G at amino acid position P315P. This amino acid is located at the second cb-EGF domain of CRELD1. The last heterozygous silent mutation was found in a
patient, changing the base pair G to A at amino acid position Q368Q which is within the transmembrane domain of CRELD1. These synonymous variants were not further characterized, although it should be noted that synonymous SNPs can affect splicing via the regulation of splice enhancers or inhibitors (Parmley et al., 2006; Nielsen et al., 2007).

Table 3.2: Synonymous and non-synonymous variants in CRELD1 found in the heterotaxy population

<table>
<thead>
<tr>
<th>Variants</th>
<th>Location</th>
<th>Functional group</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>G&gt;GT (heterozygous)</td>
<td>Promoter 2 at 9951009</td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>G&gt;T (homozygous)</td>
<td>Promoter 2 at 9951009</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>T&gt;TC (heterozygous)</td>
<td>Promoter 2 at 9950860</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>C&gt;CT (heterozygous)</td>
<td>Promoter 2 at 9950628</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>A&gt;G (homozygous)</td>
<td>Exon 1, M13V</td>
<td></td>
<td>All samples (126)</td>
</tr>
<tr>
<td>A&gt;AG &amp; A&gt;G</td>
<td>Intron 3 at 9954660</td>
<td>23% heterozygous</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>11% homozygous</td>
<td></td>
</tr>
<tr>
<td>G&gt;A (homozygous)</td>
<td>Intron 6 at 9957920</td>
<td></td>
<td>All samples (126)</td>
</tr>
<tr>
<td>C&gt;CT (heterozygous)</td>
<td>Exon 8, L304L</td>
<td>CRELD domain</td>
<td>1</td>
</tr>
<tr>
<td>GA deletion</td>
<td>Introns 8 at 9959990/1</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>G&gt;GA</td>
<td>Exon 9, P315P</td>
<td>cb-EGF domain</td>
<td>3</td>
</tr>
<tr>
<td>C&gt;CT (heterozygous)</td>
<td>Introns 9 at 9960229</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>G&gt;GA (heterozygous)</td>
<td>Exon 10 at Q368Q</td>
<td>Transmembrane</td>
<td>1</td>
</tr>
</tbody>
</table>
This study demonstrates that there are missense mutations in *CRELD1* that may act to increase the likelihood of AVSD or other CHD in heterotaxy patients. In this study three different heterozygous missense mutations in *CRELD1* were identified in three unrelated individuals from a patient population of 126 who were diagnosed with heterotaxy. Approximately 66% of the heterotaxy population had different types of heart defects (Fig. 4.1, A). The three individuals carrying mutation in *CRELD1* were diagnosed with different forms of heart defects in addition to AVSD which accounts for about 9% of patients with heterotaxy and heart defects (Fig. 4.1, B) and approximately 6.3% in total heterotaxy population used in this study. All three mutations were identified in highly conserved regions of *CRELD1* and can alter CRELD1 properties. They are suggesting that mutation in *CRELD1* may increase the susceptibility of CHD in the heterotaxy population.

**Figure 4.1:** A, Approximate ratio of heterotaxy patients diagnosed with heart defects and without heart defects in study population. B, The ratio of different types of heart defects in heterotaxy patients with heart defects found in this population.
In one case the mutation was an A to T transition in exon 5 at cDNA position 615 (c.615A>G) that results in a substitution of threonine for alanine at amino acid 206 (p.A206T) in the CRELD domain of CRELD1. This domain has extracellular function and is 333 amino acids long (p. 30-362) and contains two potential N-linked glycosylation sites one at amino acid position 79 and 205. N-linked glycosylation of some membrane proteins is critical for proper folding, co-assembly and subsequent trafficking through the biosynthetic pathway, suggesting that it is necessary for proper functional role of CRELD1. The mutation changes the amino acid located at sequon position of the second N-linked glycosylation site. The sequon position is Asn-X-Ser or Asn-X-Thr, where X is any amino acid except proline. In CRELD1 the sequon position is Asn-Ala-Ser. The mutation, A206T, changes the alanine to threonine. According to the consensus sequence X can be any amino acid except proline, but changes in the sequence of this sequon have shown to have a major effect on the ability of various tissues to utilize N-linked glycosylation signals (Nishikawa and Mizun, 2001).

Therefore this alteration would not likely abolish glycosylation, but it could affect the efficiency. Using the bioinformatics tool, PolyPhen-2, suggests that this variant is unlikely to affect the protein structure. However as can be seen in Fig.4.2, the molecular formula for alanine is C₃H₇NO₂. This amino acid is non-polar and hydrophobic and is known to be ambivalent, meaning it can be inside or outside of the protein molecule. On the other hand, threonine is slightly polar with a molecular formula of C₄H₉NO₃, and compared to alanine it has one extra carbon and hydroxyl...
group making it a hydrophilic molecule. Although Polyphen-2 indicates that this substitution will have a benign effect on protein structure, my analysis suggests that this mutation may have the potential to significantly change the protein structure.

![Chemical structures of alanine (A) and threonine (B)](image)

**Figure 4.2:** Alanine (A) and threonine (B) chemical structure.

In addition, this non-synonymous variant is located in the CRELD domain that has extracellular function and such non-conservative alteration in CRELD1 can affect its proper function. This alteration was not present in 93 race-relevant control chromosomes, indicating the novelty of this mutation and suggesting an association of risk of developing AVSD that may work in concert with addition factors such as heterotaxy in this study. Further study can be used to confirm our expectation on changes in protein structure by analyzing a recombinantly expressed mutant CRELD1 in which the physical properties of mutant protein can be compared with wild-type CRELD1.

The second mutation was a single base alteration of A to G at cDNA position 792 (c. A792G) causing amino acid substitution of alanine for threonine at amino acid position 265 (p. T265A). This SNP is within the exon 7 and located in a highly
conserved region CRELD domain with extracellular function. Fig.4.2 shows the chemical structure of these two amino acids. This amino acid change will reduce the polarity of the protein and subsequently can alter its function.

Based on the analysis using PolyPhen-2, this region is highly conserved among more than 20 different species and mutation at c.256 is predicted to have damaging effect on the protein structure. Although the specific effect of this mutation on the structure of CRELD1 is still unknown. The patient with this mutation exhibits a broad range of heart defects in heart valves and chambers in addition to TGA, AVSD, and coarctation of aorta (CoA). Race relevant population study confirmed the novelty of this mutation. This data is consistent with my hypothesis that there are mutations in CRELD1 that can increase the risk of developing CHD in heterotaxy population.

The last mutation was found in a patient with situs inversus with AVSD and asplenia. The mutation is a C to T transition in exon 9 at cDNA position 985 (c.985 C>T) that results in a substitution of cysteine for arginine at amino acid 329 (p.R329C). The chemical structure of these two amino acids is shown in Fig.4.3. The mutation is in a very highly conserved region in the second cb-EGF domain of the protein. This mutation was previously detected in individuals with non-syndromic AVSD (Robinson et al., 2003) and AVSD with Down’s syndrome (Maslen et al., 2006; Kusuma et al., 2011) and was determined to not be present in race-relevant control chromosomes indicating that it is a disease-specific mutation.
CRELD1 has two highly conserved cb-EGF domains (Fig. 3.3, B). The first cb-EGF domain is 41 amino acids long, at amino acid position 153 to 193. The second cb-EGF domain consists of 40 amino acids (p.305-344). These domains have a very specific disulfide bonding pattern which is essential for proper protein function (Robinson et al., 2003). Consequently, addition of a free cysteine residue, as occurs with this mutation, change the β-sheet in the secondary structure of CRELD1 and would be expected to interfere with protein folding (Fig. 4.4).

![Figure 4.3: Arginine (A) and cysteine (B) chemical structure.](image)

Accordingly, previous studies have shown that the p.R329C mutation alters the protein structure (Robinson et al., 2003). Furthermore, this amino acid position is conserved as an arginine residue among mammals (Fig. 3.6, B). Taken together, these data suggest that the mutation p.R329C is involved in causing CHD in heterotaxy patients.

In this study 84 out of 126 (67%) patients with heterotaxy had a broad range of heart defects. Based on this study, CRELD1 mutations had a prevalence of about...
2.4% in total heterotaxy population. Identification of *CRELD1* mutations in 3 out of 84 heterotaxy patients diagnosed with broad range of CHD suggesting the prevalence of *CRELD1* mutations appears to be approximately 3.5% in heterotaxy population with different types of heart defects. In addition, all three mutations were found in patients with heterotaxy who were also diagnosed with AVSD (3/8) suggesting a high prevalence of 37.5% in this portion of the population. These data suggest that defects in *CRELD1* may contribute to the pathogenesis of AVSD and CHD in the context of heterotaxy. This is consistent with previous studies that demonstrated that *CRELD1* mutations were associated with AVSD and heterotaxy as well as nonsyndromic AVSD (Robinson et al., 2003; Maslen, 2004). However, the high incidence of *CRELD1* mutations in individuals with heterotaxy and AVSD indicates that the heterotaxy background significantly sensitizes those individuals towards developing an AVSD since the incidence of *CRELD1*\textsuperscript{mut}-associated AVSD in other populations.
Figure 4.4: Diagrammatic representation of the two cb-EGF domain of CRELD1. The R329C mutation is indicated by the arrow. Conserved amino acid residues defining cb-EGF domains are indicated by the single letter amino acid code. Black lines show the disulfide-bonding pattern for the conserved cysteine residues (Robinson et al., 2003).

Consequently, based on these results, I conclude that CRELD1 mutations in general may increase the risk for developing an AVSD and CHD in heterotaxy patients. However, it is very important to consider that in the process of L-R asymmetry and heart development, both are following a very complicated pathway and many factors can affect their process. The question remains as to what combination(s) of factors is required to exceed the threshold causing a complete AVSD and CHD. The results of this study support the premise that mutations in CRELD1 are a risk factor for AVSD and CHD in heterotaxy population.

Previous studies suggest that combinations of rare mutations, as see in this study, in genes that control early embryonic L-R patterning and environmental factors during embryogenesis are causing most of the cases heterotaxy with heart
defects. Future works will have to focus on studying the other candidate genes such as \textit{NKX2.5} in a larger population (Belmont 2004). Such information will provide the factors effecting disease susceptibility in heterotaxy population. These can lead to the ability of early disease detection and relatively in future to therapeutic ways of restoring the function of altered genes.

**FUTURE STUDIES**

Mouse model can be used to study on how mutations in \textit{CRELD1} would increase the risk of developing AVSD and CHD in heterotaxy population. This can be done using F1 generation by crossing heterotaxy mouse \textit{Zic3}(+/-) and \textit{Creld1}(+/-) mouse and studying the rate of heart defects in their progenies. In addition, more study has to focus on looking for other unknown genes that increase the susceptibility of developing AVSD and CHD in heterotaxy.
REFERENCES


Appendix: CRELD1 genomic sequence.

acgt = R pcr primer
acgt = F pcr primer
acgt = R seq primer
acgt = F seq primer

CRELD1 Genomic

Pro1F
Pro1Seq
Pro1R
Pro1Rseq
Pro2F
Pro2Seq
Pro2R
E1Seq

E1-2F
Exon 1

57
Translation (NP_056328.2 and NP_001070883.1)

MAPWPPKGLVPAMLWGLSLFLNLPGPILWQPSPQSSPPQPQHPCHTCRGLVSFNNKGL
ERTIRDNFGGGNTAEENLSKYKSDSRILVLEVEVLEGVCSSDFSCEILRLELSEELVESWW
FHKQEQAPDLFQWLCSDSLKLCPPAGTFPSCLPCHGTPCCGYQCEGTEGRGSGH
CDCQAGGYGGEACGCGLGYFEAERNASHLVCSCACFGPACRCSGPEESNCLCQLCCKGWALHH
LKCVDIDECGTGANCQDGFCVNETEGYECRDCAKACLGCAMPGGCRKCCSPQGYQVG
SKCLDVDECETEVCPGENKQCENTEGGYRICAEGYKQMEICVKEQIPESAGFSEMTE
DELVVLQMFHGIICALATLAAKGDVFATIFIGAVAAMTGYWLSESRDVLLEGFIKGR
X