HYPOPLASTIC LEFT HEART SYNDROME:

MOLECULAR CONSEQUENCES OF TRANSCRIPTION FACTOR MUTATIONS

by

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MOLECULAR CONSEQUENCES OF TRANSCRIPTION FACTOR MUTATIONS

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ABSTRACT

Hypoplastic left heart syndrome (HLHS) is a congenital disorder affecting the left side of the heart. It is characterized by an underdeveloped left ventricle, an atrial septal defect, a narrowed aorta, and an open ductus arteriosus. Children diagnosed with HLHS generally present with cardiogenic shock, such as arrhythmia, cardiac arrest, or congestive heart failure, and are likely to die without surgical intervention. To improve diagnostic options, an understanding of the etiology of HLHS is needed. A genetic linkage to HLHS has been proposed; unfortunately, little evidence exists to support this hypothesis, as the mechanisms of cardiogenesis are only partially known. However, Holt-Oram Syndrome (HOS), a congenital disorder of heart and hand development, can present with HLHS as a symptom and has been linked to a T-box transcription factor involved in cardiac development, *TBX5*. Other studies have reported families with atrial septal defects, a symptom of HLHS, to carry mutations in *NKX2.5*, another transcription factor involved in cardiogenesis. This study hypothesized, based on previous findings, that mutations in *TBX5*, *NKX2.5* or another co-transcription factor involved in cardiogenesis contribute to the etiology of HLHS. To examine the mutational status of *TBX5* and *NKX2.5* in patients with HLHS, venous blood and atrial appendage tissue were collected for DNA isolation and polymerase chain reaction amplification of the region of interest. Genomic DNA sequencing of *TBX5* revealed an interesting combination of 11 synonymous, non-synonymous, and intronic variations among 19 probands out of 110 screened. The non-synonymous variations were all in exon 8. *NKX2.5* showed one variation in one proband out of 110.

Computer analyses determined the probability of pathogenicity of each variation but further *in vitro/in vivo* studies need to be conducted to more clearly determine the effect of these variations on cardiac development. The possible correlation between genotype and phenotype could help further the understanding of the molecular mechanisms behind cardiac and HLHS development.

Chapter 1

INTRODUCTION

1.1 Hypoplastic Left Heart Syndrome

Congenital heart malformations are the most common birth defects and are the leading cause of infant mortality within the first year of life [5]. Hypoplastic left heart syndrome (HLHS, OMIM: #241550) (Fig. 1.1) is characterized by underdevelopment (hypoplasia) of the left ventricle and aorta, narrowing (stenosis) and/or closure (atresia) of the aortic arch, atrial septal defect (foramen ovale) and an open (patent) ductus arteriosus [3].

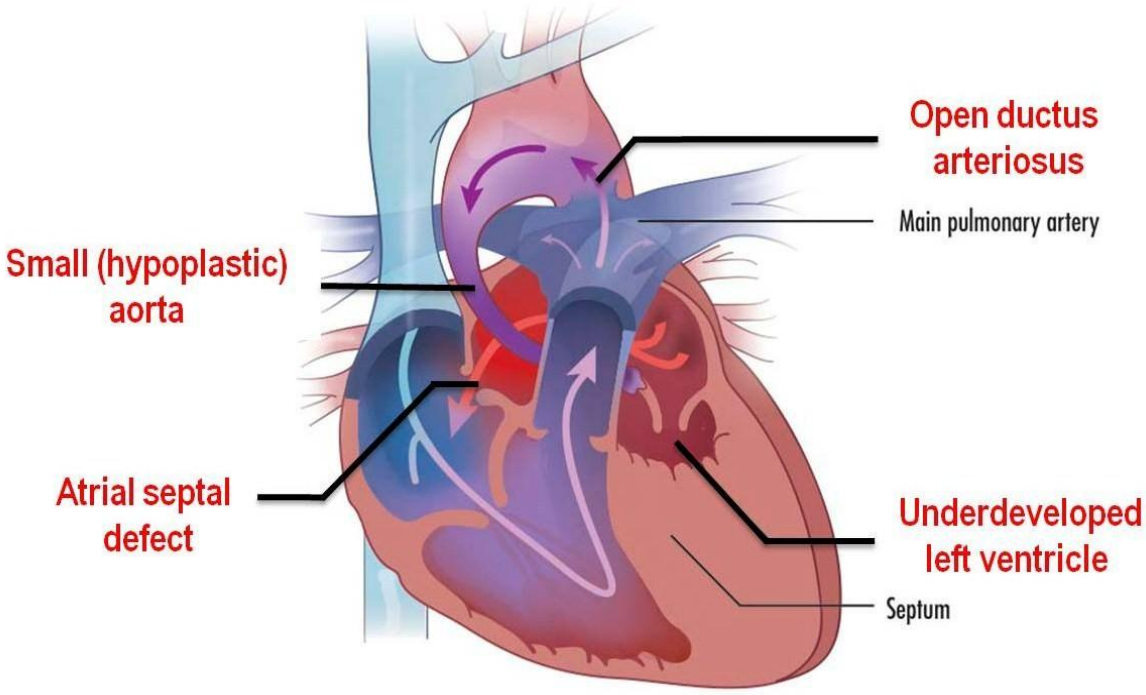

Figure 1.1: Hypoplastic Left Heart Syndrome Heart Structure. The four main defects are shown; patent ductus arteriosus, hypoplastic left ventricle, atrial septal defect and hypoplastic aorta. Blood flow indicated by direction of arrows. (http://www.childrenshospital.org/az/Site502/mainpageS502P0.html)

The ductus arteriosus and foramen ovale are common elements of fetal circulation which connect the pulmonary artery to the aorta and allow oxygenated and deoxygenated blood to mix, respectively. (Fig. 1.2)

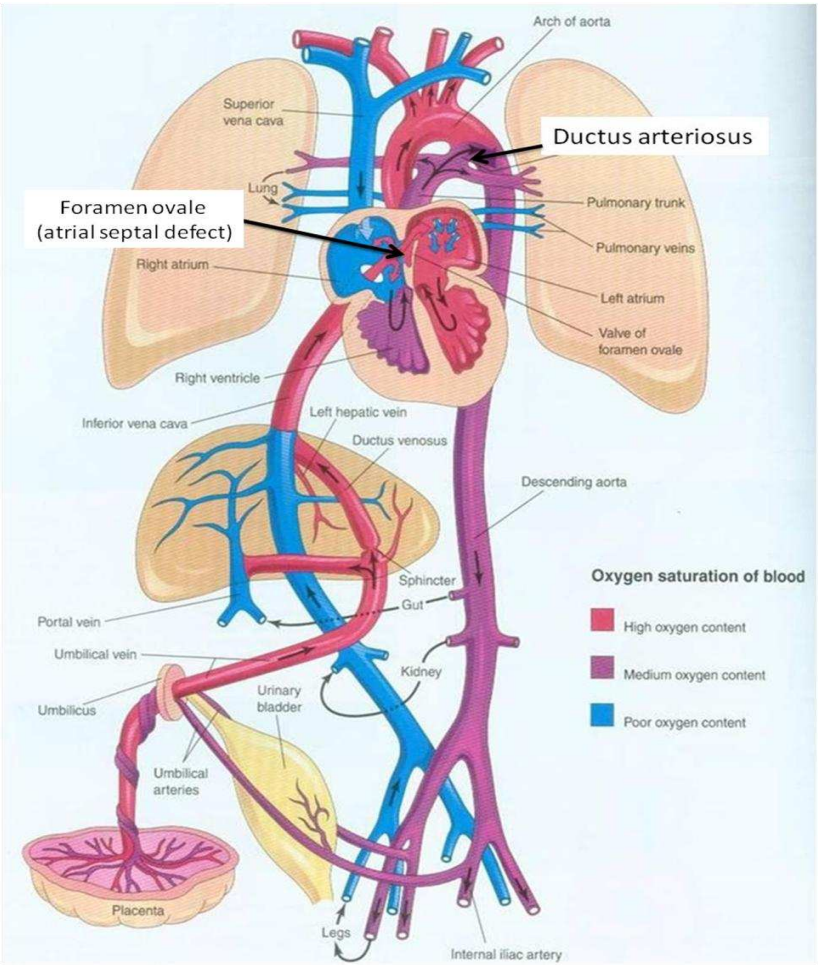

Figure 1.2: Fetal Circulation. Notice the foramen ovale and ductus arteriosus. (http://mcb.berkeley.edu/courses/mcb135e/fetalc.html)

The hypoplastic left ventricle and aorta make the left side of the heart inefficient at sustaining systemic circulation, and the atrial septal defect allows the oxygenated blood from the left side of the heart to be redirected to the right side, through the pulmonary artery and patent ductus arteriosus into the aorta and out to the body. It is common, however, for the ductus arteriosus to close within the first few days of life, causing the newborn to lose their only ability of receiving oxygenated blood to the

body. Therefore without surgical intervention, HLHS is fatal within the first few days of life.

This rare disorder occurs in about 0.16-0.36 of every 1,000 live births (www.orpha.net) and is more prevalent in males than females. HLHS comprises 1.2 - 1.5% of all congenital heart defects (CHD) or 7-9% of all CHD diagnosed within the first year of life. It is hypothesized that HLHS may be caused by mutations in *TBX5* or other cardiac-specific transcription factors. The purpose of this study is to explore the possibility of a genetic aspect to HLHS and, if one is determined, develop a diagnostic test.

1.2 Normal Cardiac Development and Function

Cardiogenesis is an elaborate process that requires spatial and temporal regulation of a multitude of cardiac as well as noncardiac-specific genes. The heart arises from mesodermal stem cells that differentiate into cardioblasts and continue to mature into cardiomyocytes that interact to create a cardiac crest consisting of two cell types (fields). These cell types have different predetermined differentiation patterns, which develop the linear heart tube and finally, the fully developed heart [17, 18]. Cardiac development requires the use of an evolutionarily conserved program involving different combinations of molecules and tissue-specific factors to differentiate mesodermal stem cells into cardiomyocytes and regulate genes involved in contractility and morphology [17]. Factors such as bone morphogenic protein

(BMP), aid in the formation of cardiomyocytes soon after gastrulation. BMP activates *NKX2.5*, the earliest cardiac lineage molecular marker, which then activates *MEF2C,* which encodes transcription factors responsible for myocyte differentiation [17].

Once specified, the cardiac muscle cells converge along the ventral midline of the embryo to form a beating linear heart tube composed of myocardial and endocardial layers, which comprise the exterior and interior layers, respectively, and are separated by an extracellular matrix [18]. In order to form the four-chambered heart, the heart tube undergoes chamber specification, septation and trabeculation [4]. Rightward looping of the heart tube generates the correct orientation of the pulmonary and systemic circulations as well as alignment with the correct vasculature [17]. The complete molecular mechanisms behind this process are currently unknown.

The developed heart (Fig. 1.3) contains the atria, which comprise the two upper chambers and are separated from each other by a fibrous partition known as the atrial septum.

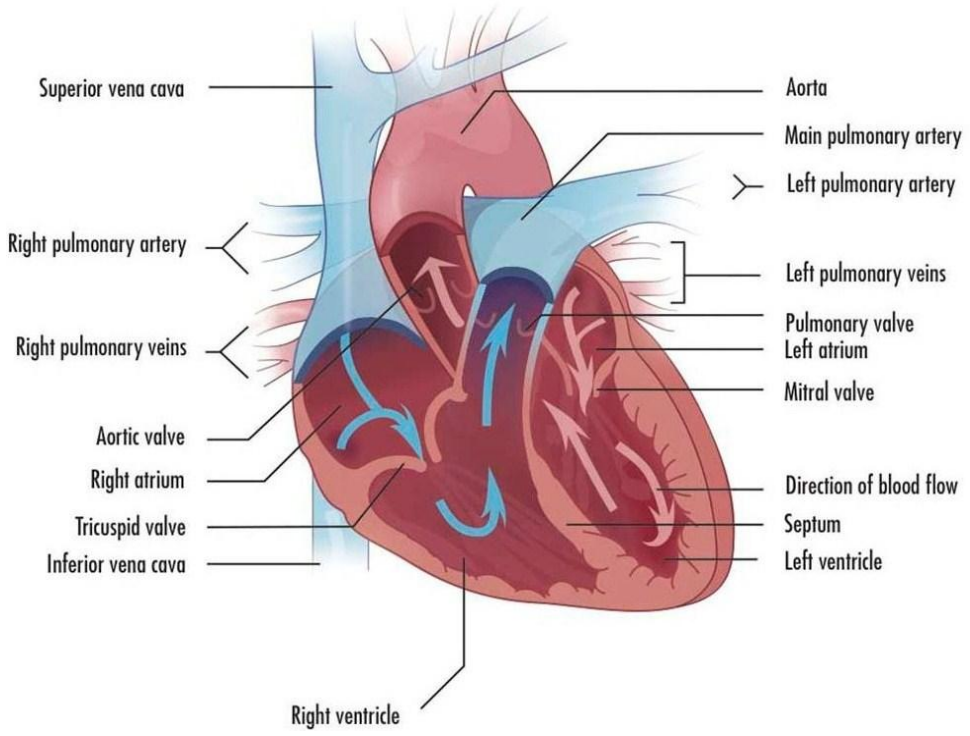

Figure 1.3: Normal Heart structure. Blood flow indicated by direction of arrows. (http://www.childrenshospital.org/az/Site502/mainpageS502P0.html)

The ventricles comprise the two lower chambers and are separated from each other by the ventricular septum. The atrio-ventricular (AV) valves, which connect the left and right atria to their respective ventricles, allow for blood to be pumped through the chambers. Blood flows from the right atrium through the tricuspid valve, into the right ventricle through the pulmonary valve, into the pulmonary artery and lungs to become oxygenated. The blood returns to the heart from the lungs through pulmonary veins and enters the left atrium. Once the blood has been pumped from the left atrium through the bicuspid valve into the left ventricle, the left ventricle provides the contractile force necessary to eject the oxygen-saturated blood through the aortic valve

into the aorta (the main artery of the body) and out to the body and through capillary beds which allows for the exchange of oxygen and nutrients for carbon dioxide and waste.

1.3 Treatment Options

Neonates with HLHS may present with cardiogenic shock, such as arrhythmia, cardiac arrest or congestive heart failure, and are likely to die without surgical intervention. While some affected infants are identified on prenatal ultrasound, some babies may appear to be healthy at birth with no detectable signs of this condition while their systemic circulation is being supported. However, many infants are only diagnosed at birth when systemic circulation fails due to the malformations, and the infant becomes profoundly cyanotic. There are two surgical interventions available to children born with HLHS, a primary cardiac transplantation (although donor hearts are scarce), or a series of staged surgical palliations to create a functionally univentricular heart. The three-staged treatment approach involves surgical interventions typically as individual operations. The Norwood procedure (stage I) allows the right ventricle to pump blood to both the lungs and body and is performed within the first few days of birth. The procedure reconstructs the hypoplastic aorta and connects it to the right ventricle through the use of the proximal main pulmonary artery, establishing systemic outflow. Then a shunt from the pulmonary artery to the aorta reestablishes pulmonary outflow [11]. (Fig. 1.4)

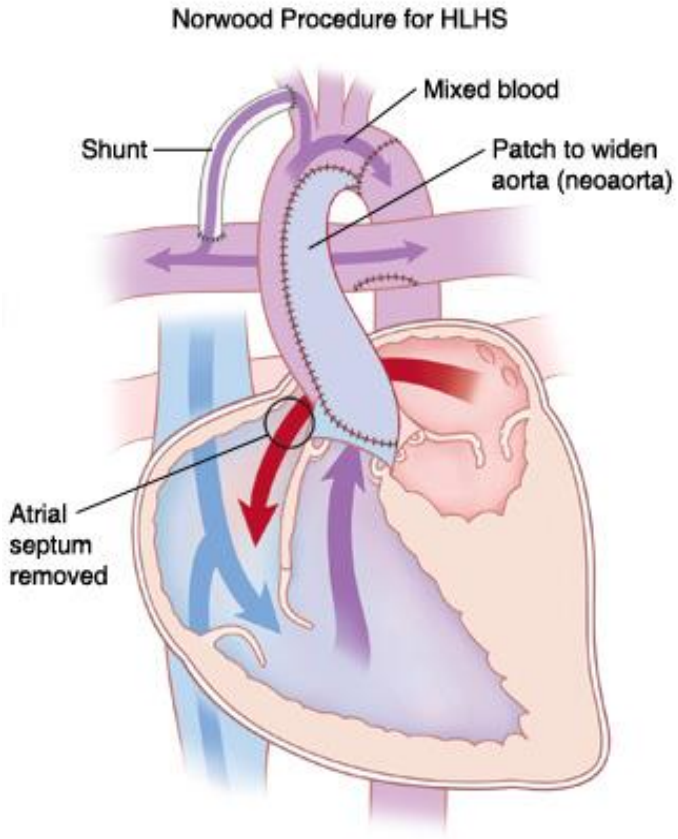

Figure 1.4: Norwood Procedure
(http://www.mountnittany.org/articles/healthsheets/6702)

The Glenn procedure or Hemi-Fontan (stage II), generally occurs between ages three and six months, removes the shunt and creates a connection between the superior vena cava and the pulmonary artery in order to supply the pulmonary blood flow [11]. (Fig. 1.5)

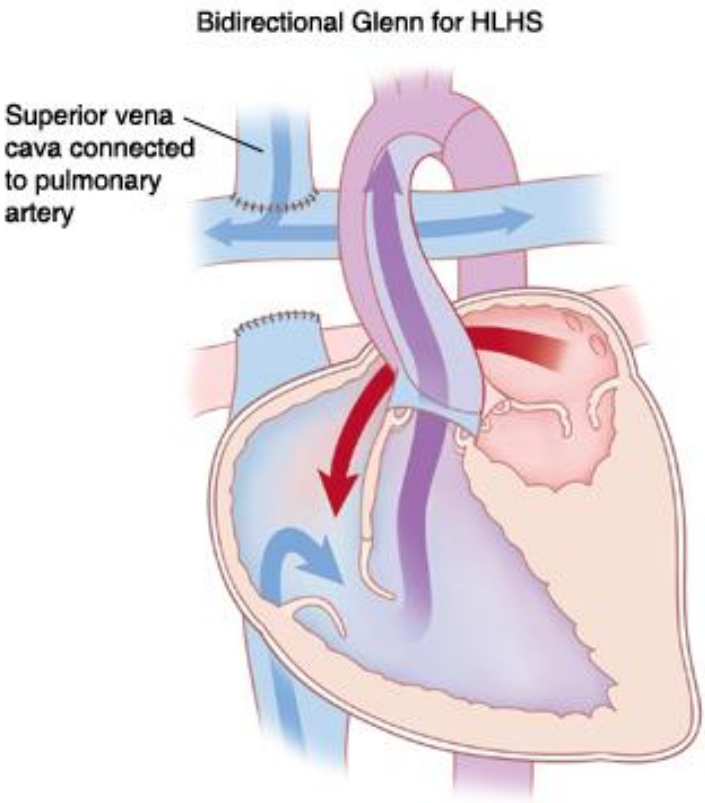

Figure 1.5: Bidirectional Glenn Procedure
(http://www.mountnittany.org/articles/healthsheets/6703)

The Fontan or Total Cavopulmonary Connection (TCPC) procedure (stage III) occurs between 18 months and 4 years of age. In this final stage of the operations, a connection is made between the inferior vena cava and pulmonary artery, therefore separating the pulmonary and systemic circulations and creating a univentricular heart [11]. (Fig 1.6)

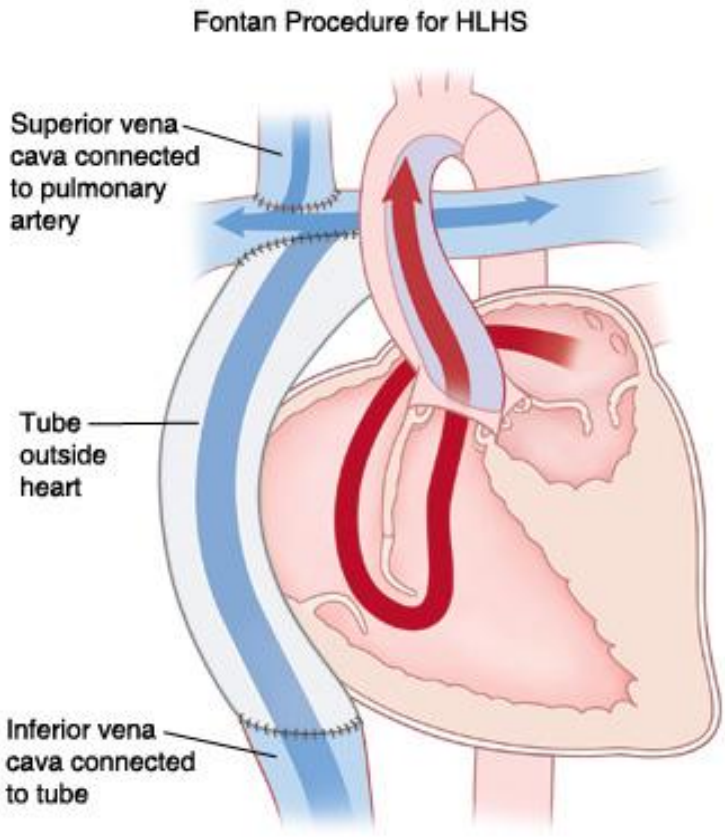

Figure 1.6: Fontan Procedure
(http://www.mountnittany.org/articles/healthsheets/6704)

The death rate (mortality) after Stage I palliation ranges from 7-35%, after the second stage between 2 and 5.4%, and after the third stage, less than 5% [3].

1.4 Congenital Heart Defects and Their Molecular Connections

Studies have indicated that HLHS has a strong genetic component [6, 12, 13, 14] with a multifactorial mode of inheritance, indicating the syndrome is not caused by one abnormal gene, but by a combination of various abnormal genes and environmental factors. The focus of this study is to screen patients with HLHS for changes in several genes associated with cardiac development, starting with *TBX5,* and *NKX2.5*. Several genetic syndromes are associated with HLHS: Turner Syndrome, trisomy 13, trisomy 18, Smith-Lemli-Opitz Syndrome, Noonan Syndrome, Jacobsen Syndrome, and Holt Oram Syndrome [3, 13, 14, 15].

Holt-Oram Syndrome (HOS; OMIM #142900) presents as an autosomal-dominant inherited condition characterized by cardiac anomalies and radial ray limb deformities, with a frequency of 1 in every 100,000 live births [7, 10]. The cardiac manifestations range from persistent left superior vena cava, the most common variation of the thoracic venous system, to HLHS. The genes affected in HOS are located on human chromosome 12q, and further elucidation revealed several distinct mutations in the *TBX5* coding sequence [7]. A majority of patients with HOS carry mutations in the T-box, DNA binding domain, of *TBX5,* resulting in reduced activation of downstream targets and interactions with co-transcription factors [2, 7]. For example, some missense mutations in the *TBX5* gene affect its protein-protein interactions and transactivation regulation activities with GATA4 and NKX2.5 [7]. Heinritz et al. reported that there was no correlation between different truncating

mutations of *TBX5* and specific clinical phenotypes of HOS, although missense mutations within the T-box domain correlated with a phenotype predominantly showing either heart or limb defects in HOS [10]. Finally, somatic mutations of *TBX5* have been identified in heart tissues of patients with congenital heart defects other than HOS [10].

NKX2.5, a co-transcription factor to *TBX5*, appears to be a commonly mutated gene that has been linked to atrial septal defects. Point mutations have been identified in *NKX2.5* in some families with atrial septal defects and cardiac conduction abnormalities [1, 17, 18, 19, 20]. Additionally, sporadic mutations of *NKX2.5* have been found in patients with tetralogy of Fallot, tricuspid valve anomalies, ventricular septal defects, Ebstein's anomaly, and subvalvular aortic stenosis [1, 17, 20]. At least 28 studies have identified and analyzed at least 41 nonsynonymous *NKX2.5* germline variations, of which only 5 of the 41 have been reported more than once in unrelated individuals with CHD [19]. The relationship between the variations and phenotype is currently unknown, however the fact that different loss-of-function mutations correlate to certain abnormalities suggests a linkage between different functional domains of NKX2.5 involved in different aspects of heart development [17].

1.4.1 *TBX5*

The T-box family of transcription factors is highly conserved, widely expressed, and promotes organ and tissue diversity. They regulate cell patterning, fate, survival, and proliferation and have clinical diagnostic potential [9]. T-box genes

frequently act in a combinatorial manner exhibiting dose-sensitivity in controlling a diverse set of developmental processes; therefore, haplo-insufficiency of a large proportion of these genes is associated with human congenital diseases [21]. With haplo-insufficiency, the total level of a protein produced by the cell is less than the normal level, causing the cell to function abnormally.

The *TBX5* (T-box protein 5) gene (OMIM #601620), first described in 1997, is a 518-amino acid member of the T-box family of transcriptional regulators and spans over 54.5kb within chromosome region 12q24.21. (Fig. 1.7)

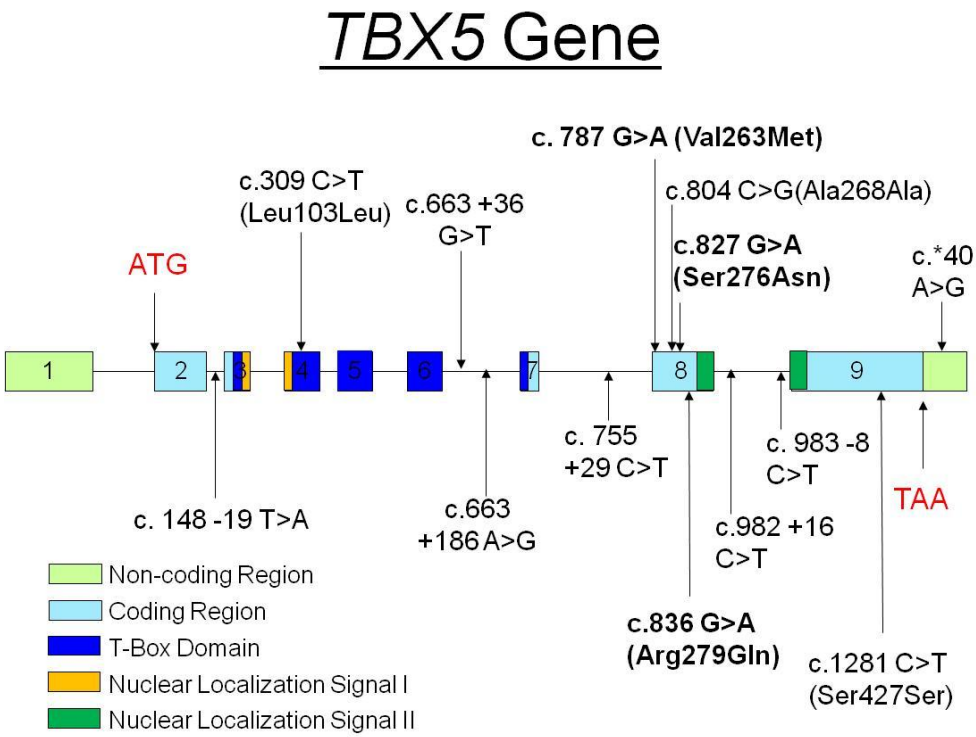

Figure 1.7: *TBX5* Gene Layout

TBX5 contains a conserved T-box DNA-binding domain (amino acids 55-237) as well as two nuclear localization signals (amino acids 78-90 and 325-340). The T-box domain is a region of conserved sequence, (182 amino acids in TBX5), which encodes a sequence specific for DNA binding. T-box proteins are transcription factors that control developmental pathways. The prototype of this family is the mouse Brachyury (or T) gene product. This highly conserved domain is a series of amino acids that recognizes and binds a specific DNA element, the T-half site. It can also bind other transcription factors, chromatin remodeling complexes, and histone modifying enzymes [21].

TBX5 is expressed in a large array of developing tissues including the liver, trachea, pharynx, lungs, forelimbs, retina, blood vessels, and heart [7, 10]. Expression of TBX5 during heart development is mainly associated with the first heart field (FHF) and is first expressed in the mesoderm and later in the endocardium/myocardium of the inflow tract, atria, atrioventricular canal and left ventricle [21]. It plays a role early in chamber differentiation and later in heart development during chamber septation. Mice homozygous for a *TBX5* null mutation establish a linear heart tube, but hypoplastic components arise from the FHF. Heterozygous mutant mice develop the HOS and show the dose-sensitivity of TBX5 to induce and maintain chamber characteristics [21]. The process that controls TBX5 expression is not well understood. TBX5 regulates the expression of the natriuretic peptide precursor A (NPPA) also referred to as atrial natriuretic factor (ANF), and GJA5, both of which play a role in the elongation of the simple cardiac tube and formation of the localized chambers.

The fact that clustered mutations in different regions of the protein cause defects in the heart as well as limbs (as in HOS) suggests that TBX5 engages different genes or cofactors, depending on the unique structural motifs in the protein [18]. TBX5 associates with diverse co-factors to activate a range of chamber myocardial genes [21]. TBX5 interacts with NKX2.5, GATA4, and TAZ to activate the *NPPA* gene during cardiac differentiation. TBX5 can also interact with SALL4 to inhibit *NPPA* gene expression and promote activation of the *GJA5* gene during cardiac septation. The *NPPA* promoter in mouse has two T-half sites to which TBX5 binds and a site for the NK-type homeobox, NKX2.5, in close proximity. Based on experiments with TBX5 deletions/point mutations, it appears the C-terminal plays a role in the association to and activation of the *NPPA* gene [8]. Cooperative binding and *NPPA* transactivation by TBX5 and NKX2.5 is aided by a third protein, GATA4 [21, 22]. Furthermore, TAZ, a transcriptional cofactor containing a PDZ-binding motif, associates TBX5 with histone acetyltransferases p300 and p300/CBP-associated factor, increasing the accessibility of the promoter and activation of *NPPA* [21]. Finally, SALL4, which is expressed in the myocardium of the interventricular septum and left ventricle, inhibits *NPPA* activation when associated with TBX5 but promotes *GJA5* activation.

1.4.2 *NKX2.5*

The *NKX2.5* (NK2 homeobox 5) gene (OMIM #600584) is a 324-amino acid member of the NK-2 family of homeodomain-containing transcription factors and spans over 3.25 kb within chromosome region 5q35.1. (Fig. 1.8)

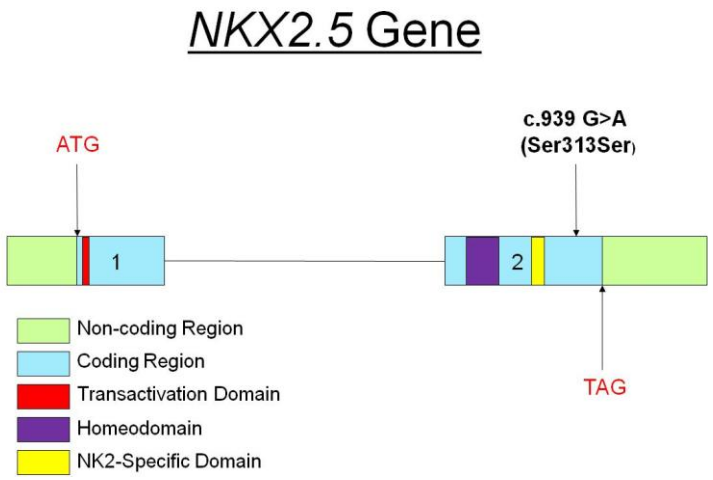

Figure 1.8: *NKX2.5* Gene Layout

NKX2.5 binds DNA using a 60-amino acid long homeodomain displaying a helix-turn-helix DNA binding motif containing three α-helices, with helix three providing binding specificity [19]. Also important for function are the transactivation domain and the NK2-specific domain [19].

NKX2.5 is an evolutionarily conserved cardiac transcription factor expressed in mesenchymal cells, and FHF and SHF, and is required for chamber and conduction system formation, maturation and maintenance [19, 21]. During development, it is the earliest molecular marker of the cardiac lineage and is expressed in the heart muscle lineage throughout life [1, 17]. NKX2.5 has been implicated to be responsible for the number of cardiac myocytes available during development through the regulation of a homeodomain-only protein, HOPX, which is part of the NKX2.5 and GATA4 negative feedback loop [18, 20]. The signaling pathway by which NKX2.5 is involved in myocyte differentiation is through activation by bone morphogenetic protein (BMP), which is secreted from endoderm, and then by activating MEF2C, which

encodes transcription factors involved in myocyte differentiation [17]. As described above, NKX2.5 can also interact with TBX5 and GATA4 to regulate the *NPPA* gene [21, 22]. The N-terminal domain and N-terminal region of the TBX5 T-box, and the homeodomain of NKX2.5 are the critical protein domains for formation of the protein complex [8]. The interaction between NKX2.5 and GATA4 occurs through the C-terminal zinc finger of GATA4 and C-terminal homeodomain of NKX2.5. GATA4 binds the auto-repressive domain of NKX2.5 located at its C-terminus, and possibly causing a conformational change revealing NKX2.5 activation domains [23]. NKX2.5 and GATA4 co-regulate expression through a mutually reinforcing positive feedback loop [17]. Finally, NKX2.5, TBX5, and GATA4 form a complex that regulates myosin heavy chain, which is needed for proper cardiac septation [18]. It is currently unknown which septal genes are regulated by these factors but mutations in the human alpha-myosin heavy chain (*MYH6*) gene, a direct target of the NKX2.5/TBX5/GATA4 complex lead to atrial septal defects [18].

Aside from interacting with TBX5 and GATA4, NKX2.5 is a regulator of HAND1 expression. HAND1 expression is elevated in the left ventricle and depends on NKX2.5; therefore NKX2.5 is critical for the FHF [18]. Mice lacking NKX2.5 present with lethal defects in the ventricles and fail to express eHAND (mouse HAND1), suggesting that eHAND may act downstream/regulated by NKX2.5 [17].

Chapter 2

MATERIALS AND METHODS

2.1 Previous Work on HLHS Project

This particular project was developed and begun in 2001 as a Nemours funded grant under Dr. Funanage as Principal Investigator, and Dr. Christian Pizarro, Susan Kirwin, and Carol Prospero as co-investigators. My role was to amplify and sequence approximately half of the samples as well as some earlier samples that needed to be redone for *TBX5* and *NKX2.5*. All data were organized, compiled and analyzed through a variety of computer programs listed below.

2.2 Human Subjects

All research studies involving human subjects conducted at Nemours were submitted for review by the Institutional Review Board (IRB) and approved by the Nemours IRB.

To explore whether variations in known cardiac developmental genes are prevalent in the HLHS population, information and samples were collected from 130 patients (Table 2.2.1); DNA from 110 patients was used in this analysis. Venous blood and right atrial tissue, from routinely discarded surgical tissue, were collected.

Table 2.2.1 Proband Demographics

Diagnosis	Males	Females	Total Patient Samples
HLHS *	46	32	78
HLHS Variant	17	14	31
Percentage of samples classified as HLHS	42%	29%	71%
Percentage of samples classified as HLHS Variant	16%	13%	29%

*Hypoplastic Left Heart Syndrome
**Demographic information is missing for proband #75

2.3 DNA Isolation

Peripheral blood was collected in EDTA tubes at the time of the first surgical intervention. Genomic DNA was extracted from 2-3 mL of peripheral blood leukocytes by using the Perfect Pure DNA isolation kit (5 Prime Inc, Gaithersburg, MD).

2.4 PCR Amplification

The coding regions and exon-intron boundaries (100-150 base pairs on each side of the exon) of the genes of interest were amplified by PCR using 200ng of genomic DNA. Primers for *TBX5* and *NKX2.5* are listed in Table 2.4.1 and Table 2.4.2, respectively, along with expected product sizes. PCR amplification conditions were as summarized in Table 2.4.3. The PCR primers and method used were previously developed by the Molecular Diagnostics Laboratory.

Table 2.4.1 *TBX5* PCR Primers

Exon	Primer Name	Primer Sequence (5' to 3')	Product Size (bp)
2	ex 2F2	CAAACTGCTCCCTCCTGTCACTAG	339
	ex 2R2	CCAGACTCTGACTTTGATCTCTGC	
3	ex 3F	GGAGGAGCAGTCTCTGTGTTTTGG	262
	ex 3R	CCAAGCCACCTTTTCTTCTTCACC	
4	ex 4F	AAGGGAAACCCGGGATGGATCTTG	318
	ex 4R	AGGTTCCACTTTTCTCTCTCCCCG	
5	ex 5F	TCATCAACAACCCTCACCTGGTGC	320
	ex 5R	AGAAACCCAGTGAGAAGAAGGAG	
6	ex 6F	AACAAGGCGAATTTAGAGGGCGGG	468
	ex 6 R2	GGTGCATATGTGTGGTGGTGACTG	
7	ex 7F	AGCTCATGTCCTGAGGTGGTCTTG	218
	ex 7R	GGGTAATTTGAGGGGTATGTGGGG	
8	ex 8F	TCTGTATCAGGGCACTGATAGGCG	340
	ex 8R	CCCCAACCCAAGGAAAGGAAAAGG	
9	ex 9F	CTGTCTCCACTTTTAGCTGCCTGG	715
	ex 9R	GGAAATGTCTGTTGTGAAGCAGGC	

Table 2.4.2 *NKX2.5* PCR Primers

Exon	Primer Name	Primer Sequence (5' to 3')	Product Size (bp)
1	ex F1	GGTGACACGAAACTGCTCATCGCT	645
	ex R2	GAGTTTCTTGGGGACGAAAGCGAC	
2	ex F3	CGAGGATCCCTTACCATTACTGTG	925
	ex R4	CTGCATAATCGCCGCCACAAACTC	

Table 2.4.3 PCR Amplification Conditions

Gene	Initial Denaturation		# of Cycles	Denaturation		Anneal		Extension		Final Extension	
	°C	Time		°C	Time	°C	Time	°C	Time	°C	Time
TBX5	94	3:00	30	94	0:30	55	0:30	72	0:45	72	7:00
NKX2.5	97	5:00	27	97	0:30	63	0:30	72	1:00	72	3:00

2.5 Gel Electrophoresis

PCR amplification products and the molecular weight standard exACTGene 100 bp PCR DNA ladder were run on a 2% agarose gel. Ethidium bromide staining and UV illumination were used to visualize the DNA fragments.

2.6 Sequencing

Following gel electrophoresis, PCR products were cleaned and prepared for sequencing using the Qiaquick PCR Purification Kit (Qiagen Inc, Valencia, CA) according to the manufacturer's instructions. Mutation screening was performed by bidirectional sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA). Sequencing reaction products were electrophoretically separated on an Applied Biosystems 3130xL Genetic Analyzer (Nemours/AIDHC Biomolecular Core Laboratory). All chromatograms were visually inspected for heterozygous sites prior to alignment to a reference sequence using MacVector and Assembler software (MacVector Inc, Cary, NC). The nucleotide numbering was based on the NCBI sequences NG_007373.1 and NG_013340.1. The sequencing method and primers used were developed by the Molecular Diagnostics Laboratory.

2.7 Restriction Enzyme Assays

In patients with observed variations in *TBX5,* particularly in exon 8, a restriction fragment length polymorphism technique was utilized to analyze the patients as well as controls. Specifically, the PCR products were digested with either *Sac*I (Fig. 2.7.1), *Tse*I (Fig. 2.7.1), or *Nla*III (Fig 2.7.2) depending on the location of the variation. The restriction enzyme assays used were developed during the course of this project.

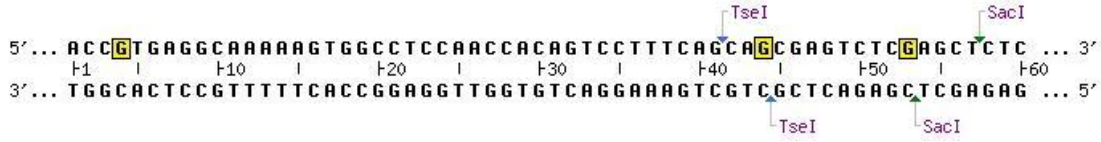

Figure 2.7.1 *Tse*I and *Sac*I Restriction Enzymes Cut-sites. Normal DNA is digested by *Tse*I and *Sac*I while *Nla*III only digests at the site created by the nucleotide change. Highlighted boxes indicate the nucleotides that were changed. (http://tools.neb.com/NEBcutter2/index.php)

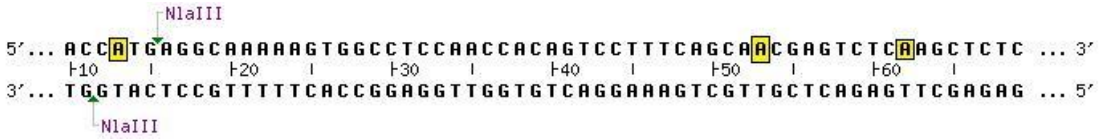

Figure 2.7.2 *Nla*III Restriction Enzyme Cut-site. Exon 8 variations found in cohort. *Nla*III cuts while *Tse*I and *Sac*I do not. Highlighted boxes indicate the nucleotides that were changed. (http://tools.neb.com/NEBcutter2/index.php)

*Tse*I digestion was carried out at 65°C for 1 hour, *Sac*I at 37°C for 1 hour and *Nla*III at 37°C for 45 minutes. Digests were analyzed through gel electrophoresis. *Tse*I and *Sac*I will cut at the sequences GC(A/T)GC and GAGCTC, respectively, whereas the bases change in the variations in probands 28 and 64, respectively, eliminates the recognition sites. *Nla*III will not digest the control DNA, but the variation in proband 6 creates a new recognition site with the sequence CATGA. Patient 6 was compared to 46 control DNA samples, patient 28 to 25 controls, and patient 64 to 100 controls to determine allelic frequencies within the general population. The control DNA samples were purchased from Sigma-Aldrich (Human Random Control DNA Panel 1) and were of United Kingdom Caucasian genetic background with a few DNA's from deidentified laboratory controls.

2.8 Functional Analysis Based on Computer Predictions

Information was gathered from five different websites to determine the effects the reported variations potentially have on protein structure, function as well as alternative splicing. Whatever information was not attained from http://www.ncbi.nlm.nih.gov/snp/ or http://useast.ensembl.org/index.html was calculated by entering the normal DNA sequence followed by the variant sequence into an online computer program.

The first program used, Sorting Intolerant From Tolerant (SIFT) (http://sift.bii.a-star.edu.sg/), predicts whether an amino acid substitution affects protein function based on sequence homology and the physical properties of amino acids. SIFT can be applied to naturally occurring non-synonymous polymorphisms and laboratory-induced missense mutations.

Polymorphism Phenotyping (PolyPhen) (http://genetics.bwh.harvard.edu/pph/), is a tool that predicts the possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations.

The online program from the Berkeley Drosophila Genome Project (http://www.fruitfly.org/seq_tools/splice.html) was used to calculate the possibility of the SNPs causing alternative splicing.

Chapter 3

RESULTS

3.1 Results for *TBX5* Exons

The exon variations detected in the HLHS study population are listed in Table 3.1. Two HLHS-affected individuals and one HLHS-Variant affected patient were found to have a non-synonymous variation in *TBX5* exon 8: Val263Met, Ser276Asn, and Arg279Gln, whereas five HLHS-affected individuals and three HLHS-Variant individuals had a synonymous variation within different regions of *TBX5*. Based on the SIFT predictions, all of the variations are tolerated, allowing for normal protein structure. Based on the PolyPhen predictions, the Val263Met mutation is potentially damaging to protein structure as well as function, while the Ser276Asn and Arg279Gln variations are predicted to be benign.

Post-PCR samples from 46 control DNAs were digested by *Nla*III, 25 controls by *Tse*I, and 100 controls by *Sac*I to determine if these exon 8 non-synonymous variations were in non-HLHS individuals. None of the controls showed a *TBX5* gene variation (data not shown).

Table 3.1: *TBX5* Exon Results

Study Number	Gender	Variation	dbSNP Variation ID	Variation Type	Codon	Effect on Protein Function°	Effect on Protein Structure and Function°°	Diagnosis
44	M	c.309 C>T Leu103Leu (Exon 4)	rs28730763	Synonymous	CTC CTT	Tolerated	----	HLHS (aortic atresia, mitral atresia)
84	F							Variant (unbal CCAVC/LV* dominant {S,D,S}** subaortic stenosis, aortic arch hypoplasia, overriding TV***, atroventricular canal type ventricular septal defect)
115	M							HLHS (aortic atresia, mitral hypoplasia)
120	M							HLHS (aortic atresia, mitral hypoplasia)
6	M	c.787 G>A Val263Met (Exon 8)	rs147405081	Non-synonymous	GTG ATG	Tolerated	Probably Damaging	Variant (critical aortic valve stenosis, mitral valve stenosis, coarctation of the aorta w/ hypoplastic transverse aortic arch)
79	F	c.804 C>G Ala268Ala (Exon 8)	rs35110399	Synonymous	GCC GCG	Tolerated	----	Variant (DORV****, cor triatriatum, hypoplastic aortic arch)
28	F	c.827 G>A Ser276Asn (Exon 8)	rs147977741	Non-synonymous	AGC AAC	Tolerated	Benign	HLHS (aortic atresia, mitral atresia)
64	M	c.836 G>A Arg279Gln (Exon 8)	rs115178276	Non-synonymous	CGA CAA	Tolerated	Benign	HLHS (aortic stenosis, mitral stenosis)
14	M	c.1281 C>T Ser427Ser (Exon 9)	rs6489957	Synonymous	TCC TCT	Tolerated	----	HLHS (mitral and aortic hypoplasia, small left ventricle)
27	M							Variant (tricuspid atresia, ventricular septal defect, partial anomalous pulmonary venous connection, s/p PA band*****, s/p repair of PAPVC******)
111	F							HLHS (aortic atresia, mitral atresia)

°Based on SIFT prediction
°°Based on PolyPhen prediction
* Unbal CCAVC/LV: unbalanced complete common atrioventricular canal defect/left ventricle
** Dominant {S, D, S,}: situs solitus, dextro, situs solitus
*** TV: tricuspid valve
**** DORV: Double outlet right ventricle
***** s/p PA band: Status post pulmonary artery banding
****** s/p repair of PAPVC: Status post repair of partial anomalous pulmonary venous connection

3.2 Results for *TBX5* Introns

Several single nucleotide polymorphisms (SNPs) were identified in eight HLHS-affected individuals: c.148 -19 T>A, c.663 +36 G>T, c.663 +186 A>G, c.755 -29 C>T, c.982 +16 C>T, c.983 -8 C>T and c.*40 A>G. Three were within female probands, and five were within male probands. Variation identification was obtained from the NCBI SNP database. (Table 3.2)

Table 3.2 Examples of *TBX5* Intron Variations

Study Number	Gender	Variation	dbSNP Variation ID	Alternative Splicing	Diagnosis
38	F	c.148 -19 T>A	rs80026530	No	HLHS (mitral hypoplasia, aortic hypoplasia, posterior malalignment ventricular septal defect)
44	M				HLHS (aortic atresia, mitral atresia)
115	M				HLHS (aortic atresia, mitral hypoplasia)
114	M	c.755 -29 C>T	Not reported	No	HLHS (aortic atresia, mitral atresia with supracardiac totally anomalous pulmonary venous connection)
13	M	c.982 +16 C>T	rs28730762	No	HLHS (aortic valve atresia, mitral valve hypoplasia)
112	F				VARIANT (critical aortic stenosis, mitral hypoplasia, left ventricle hypoplasia, hypoplastic aortic arch and ascending aorta)
108	F	c.983 -8 C>T	Not reported	No	HLHS (aortic hypoplasia, mitral atresia)
1	M	c.*40 A>G	rs10850326	No	HLHS (mitral hypoplasia, aortic atresia, hypoplastic left ventricle, ascending aorta: 4mm)

3.3 *TBX5* Hardy-Weinberg Results

The allelic frequencies for each exonic and intronic variation were calculated through the Hardy-Weinberg equilibrium equation and the results were compared to population frequencies from the NCBI SNP database. (Tables 3.3, 3.4) A chi-squared test for goodness-of-fit analysis was used to determine if there were significant differences between the allele frequencies in the HLHS population compared to the NCBI dbSNP control population. Two variations were significantly different from those of the control population, c.663 +36 G>T and c.*40 A>G.

Table 3.3 Hardy-Weinberg Allele Frequencies for *TBX5* Exon Variations

Variation	Ancestral Allele	Genotype	# with Genotype	Genotype Freq.	Allele	Allele Freq.*	Pop. Freq.**	Chi-Squared
c.309 C>T (Exon 4)	C	CC	106	0.955	C	0.977	0.942	3.71
		CT	5	0.045	T	0.023	0.058	
		TT	0	0				
c.787 G>A (Exon 8)	G	GG	110	0.991	G	0.995	0.994	0
		GA	1	0.009	A	0.005	0.006	
		AA	0	0				
c.804 C>G (Exon 8)	C	CC	110	0.991	C	0.995	0.998	1.00
		GC	1	0.009	G	0.005	0.002	
		GG	0	0				
c.827 G>A (Exon 8)	G	GG	110	0.991	G	0.995	1.000	1.00
		GA	1	0.009	A	0.005	0	
		AA	0	0				
c.836 G>A (Exon 8)	G	GG	110	0.991	G	0.995	0.999	1.00
		GA	1	0.009	A	0.005	0.001	
		AA	0	0				
c.1281 C>T (Exon 9)	C	CC	108	0.973	C	0.986	0.975	1.02
		CT	3	0.027	T	0.014	0.025	
		TT	0	0				

* Frequency from HLHS proband population (110 Patient Samples)
** Frequency from NCBI SNP database (Numbers based on: ESP_Cohort_Populations)

Table 3.4 Hardy-Weinberg Allele Frequencies for *TBX5* Intron Variations

Variation	Ancestral Allele	Genotype	# with Genotype	Genotype Freq.	Allele	Allele Freq.*	Pop. Freq.**	Chi-Squared
c.148 -19 T>A (Intron 2)	T	TT	108	0.973	T	0.986	0.996[a]	1.01
		AT	3	0.027	A	0.014	0.034[a]	
		AA	0	0				
c.663 +36 G>T (Intron 6)	G	GG	22	0.198	G	0.387	0.630[b]	26.28
		GT	42	0.378	T	0.613	0.370[b]	
		TT	47	0.423				
c.663 +186 A>G (Intron 6)	A	AA	68	0.613	A	0.712	0.635[b]	2.96
		GA	22	0.198	G	0.288	0.365[b]	
		GG	21	0.189				
c.755 +29 C>T (Intron 7)	C	CC	110	0.991	C	0.995	No data available	-----
		CT	1	0.009	T	0.005		
		TT	0	0				
c.982 +16 C>T (Intron 8)	C	CC	109	0.982	C	0.991	No data available	-----
		CT	2	0.018	T	0.009		
		TT	0	0				
c.983 -8 C>T (Intron 8)	C	CC	110	0.991	C	0.995	No data available	-----
		CT	0	0	T	0.005		
		TT	1	0.009				
c.*40 A>G (3' UTR)	A	AA	110	0.991	A	0.995	0.692[c]	77.03
		GA	1	0.009	G	0.005	0.308[c]	
		GG	0	0				

* Frequency from HLHS proband population (110 Patient Samples)

** Frequency from NCBI SNP database (Numbers based on: pilot_1_YRI_low_coverage_panel[a], JBIC-allele[b], Pilot_1_CHB+JPT_low_coverage_panel[c])

3.4 Results for *NKX2.5* Exons

The *NKX2.5* exonic variation detected in the HLHS study population is listed in Table 3.5.1. One HLHS-affected individual had a synonymous serine to serine change.

Table 3.5.1 *NKX2.5* Exon Variation

Study Number	Gender	Variation	dbSNP Variation ID	Variation Type	Codon	Effect on Protein Function*	Effect on Protein Structure and Function**	Diagnosis
14	M	c.939 G>A Ser313Ser (Exon 2)	Not Reported	Synonymous	TCG TCA	Tolerated	----	HLHS (mitral and aortic hypoplasia, small left ventricle)

*Based on SIFT prediction
**Based on PolyPhen prediction

The allele frequencies for the variation were calculated through the Hardy-Weinberg equilibrium equation. Population frequencies were not available for comparison from the NCBI SNP database.

Table 3.5.2 Hardy-Weinberg Allele Frequencies for *NKX2.5* Variation

Variation	Ancestral Allele	Genotype	# with Genotype	Genotype Freq.	Allele	Allele Freq.*	Pop. Freq.**
c.939 G>A (Exon 2)	G	GG	110	0.991	G	0.995	No data available
		GA	1	0.009	A	0.005	
		AA	0	0			

* Frequency from HLHS proband population (110 Patients Samples)
** Frequency from NCBI SNP database (None available)

Chapter 4

DISCUSSION

4.1 *TBX5*

The study population exhibited a mix of *TBX5* synonymous, non-synonymous, and intronic variations. It is of interest that of the 13 *TBX5* variations, three non-synonymous variations are clustered in exon 8, which is located in a domain necessary for *NPPA* gene binding and activation [8]. The first non-synonymous change is from nonpolar valine (R=-CH-CH3-Ch3), position 263, to nonpolar methionine (R=-CH2-CH2-S-CH3). The next non-synonymous change is from polar serine (R=-CH2-OH), position 276, to polar asparagine (R=-CH2-C=O-NH2). The final documented non-synonymous change is from basic arginine (R=-CH2-CH2-CH2-NH-C=NH2+-NH2), position 279, to polar glutamine (R=-CH2-CH2-C=O-NH2). The difference in amino acid side chains can potentially affect the formation of weak interactions during protein folding, causing destabilization to the native conformation of *TBX5*, leading to loss of proper functionality [26, 27]. Improper folding can potentially lead to haplo-insufficiency, protein degradation, or confer the ability to interact with different proteins [27].

Faria et al. [25] reported a V263M mutation in *TBX5* in a Brazilian family diagnosed with atrial septal defects and extremity anomalies. This paper focuses on cardio-skeletal anomalies as opposed to solely cardiac deformities. The affected proband in our HLHS study did not present with an atrial septal defect but does have a range of other cardiac abnormalities including critical aortic valve stenosis, mitral

valve stenosis, and coarctation of the aorta with a hypoplastic transverse aortic arch. Unfortunately, our HLHS study does not have detailed information of family histories, making determination of inheritance difficult. The family studied by Faria et al. [25] had a large number of individuals affected in the first generation suggesting an autosomal dominant pattern of inheritance. However, investigation of the parents revealed an unaffected mother and a father with negligible cardiac symptoms and no skeletal abnormalities. Upon molecular diagnosis of *TBX5,* it was shown that he was a carrier of a *TBX5* mutation also present in his three daughters, who displayed complete penetrance of the syndrome. Also, only a small number of individuals in the second generation were affected with cardiac anomalies. This makes it difficult to determine a genotype-phenotype correlation between V263M and cardiac defects.

In the present HLHS study population, there were three synonymous variations, Leu103Leu, Ala268Ala and Ser427Ser. Synonymous changes can have an impact on translation efficiency since cells usually have different amounts of tRNA for the different codons specifying a particular amino acid [16]. When a synonymous change occurs, it requires binding a different tRNA. If this tRNA is under- or over-represented in the pool of tRNAs specifying a particular amino acid, the elongation rate can potentially decrease or increase, resulting in different levels of protein [26, 27].

Porto et al. [24] reported an A268A (c.804 C>G) change in a female patient with an atrial and ventricular septal defect, mitral valve insufficiency, dilation of left atrium and pulmonary artery, preaxial polydactyly of the right thumb with duplication of the distal phalanges, distal implantation of the left thumb, mild hemithorax hypoplasia, upper-limb supination limited to the right side, and a sacralized transition

vertebra. The mutation is not expected to be the cause of typical HOS and was not found in the 100 control chromosomes tested in the Porto et al. study. They also reported a Leu103Leu (c.309 C>T) change in three female patients. The first patient presented with an atrial septal defect and triphalangeal thumbs, the second with radionular synostosis of the left arm, hypoplasia of the distal and middle phalanges of the first and fifth digits, synostosis of ulna and humerus, a hypoplastic radius, two digital rays of the right hand and no congenital heart diseases, and the third with an isolated heart defect of the Ostium secundum-atrial septal defect type.

The rest of the variations detected in this study were located in intronic regions possibly causing alternative splicing which can lead to skipped exons, mRNA degradation, exon rearrangement and inclusion of intron-encoded sequences into the protein [26, 27]. The possibility of alternative splicing was determined using an online splicing tool. According to this tool, none cause alternative splicing; however, computers cannot mimic the processes that occur *in vivo*, and therefore further *in vitro/in vivo* testing would have to be done to understand the changes further.

Future proposed work includes taking *TBX5/NKX2.5* negative samples and screening other genes of interest (*GATA4* or *HAND1*) to determine their mutational status. This would be done in the same manner as described in this paper. Another possible option is to develop expressions plasmids containing the variations described in this paper in order to generate the protein and use co-immunoprecipitation to observe if the variation affects the protein's ability to bind to co-transcription factors. Finally, another direction discussed was to generate a Zebrafish model with the discussed variations and observe the heart development of the fish to determine if the variations have an effect on cardiogenesis.

4.2 *NKX2.5*

Although the variation in *NKX2.5* is synonymous, the number of tRNAs available for polypeptide synthesis changes as a result of the variation. The tRNA corresponding to the TCG (wild-type) codon is underrepresented, whereas the tRNA for the TCA (variation) codon is a more numerous, potentially allowing faster incorporation of serine into the polypeptide chain. This, in turn, could have an effect on the elongation rate and expression level [16].

4.3 Genotype-Phenotype Correlation

There are a few factors relating to the difficulties in recognizing the genetic causes of congenital heart diseases, including genetic heterogeneity, reduced penetrance, variable expressivity, and allelic expression variability. Genetic heterogeneity exists when similar morphologic and functional phenotypes are caused by more than one gene; for example tetralogy of fallot (TOF) has at least five genetic causes: *NKX2.5* mutations, trisomy 21, deletion of chromosome 22q11, *JAG1* mutations and other cytogenetic abnormalities [28]. With reduced penetrance, an individual appears normal, but carries a disease-causing mutation [28]. Variable expressivity is indicated when individuals with the same genetic defect have varied phenotypes; for example, mutations in *NKX2.5* have been linked to atrial septal defects, TOF, Ebstein's anomaly, and ventricular septal defects [28]. Finally, there can be allelic expression variability in which the "normal" allele, depending on SNPs, could show variable expressivity [28].

In summary, genetic variations in the *TBX5* and *NKX2.*5 genes were detected in a subset of HLHS patients. When referring back to the patient results and looking over the diagnosis of each patient, there is a wide variety of phenotypes classified as

HLHS. Given the fact that cardiogenesis is such a highly spatially and temporally regulated process, these findings support the hypothesis that HLHS is a multifactorial syndrome that shows a high degree of variable penetrance. In addition, this study shows an interesting cluster of variations within exon 8 of the *TBX5* gene. This domain of the protein interacts with other transcription factors that regulate cardiac development, and suggests a potential mechanism by which genetic alterations in key cardiac transcription factors lead to HLHS [8].

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