Cytogenetic and Molecular Genetic Evaluation of Congenital Anomalies with Special Emphasis to Congenital Heart Diseases

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In

Cytogenetics and Molecular Genetics

Under the Faculty of Allied Health Sciences

By

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I, **Divya C,** do hereby declare that the research work presented in the thesis titled **"Cytogenetic and Molecular Genetic Evaluation of Congenital Anomalies with Special Emphasis to Congenital Heart Diseases"** is an original work carried out under the Supervision of Dr. A.V.Moideen Kutty, Professor, Department of Cell Biology & Molecular Genetics and under Co-supervisions of Dr. Mitesh Shetty, Department of Cell Biology and Molecular Genetics, Sri Devaraj Urs Academy of Higher Education & Research and Dr. J. Krishnappa, Professor, Department of Pediatrics, R.L. Jalappa Hospital and Research Centre teaching hospital of Sri Devaraj Urs Medical College for the award of the Degree of Doctor of Philosophy and that no part of this work has previously formed the basis for the award of any Degree, Diploma, Fellowship or any other similar title or recognition.

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Divya C

ABBREVIATIONS

8-OHdG - 8-hydroxy-2'-deoxyguanosine

Array CGH - Array Comparative Genomic Hybridization

AS - Aortic Stenosis

ASD - Atrial Septal Defect

AVSD - AtrioVentricular Septal Defect

CA - Congenital Anomalies

CHD – Congenital Heart Disease

COA - Coarctation Of the Aorta

DAPI - 4′,6-diamidino-2-phenylindole

dNTPs - deoxyribonucleotide triphosphate

DORV - Double Outlet Right Ventricle

EA - Ebsteins Anomaly

ECD – Endocardial Cushion Defect

EDTA - EthyleneDiamineTetraacetic Acid

ELB - Erythrocyte Lysis Buffer

ELISA – Enzyme Linked ImmunoSorbent Assay

ESE - Exonic Splicing Enhancer

ESS - Exonic Splicing Silencer

FISH - Fluorescence *in situ* hybridization

HCl – Hydrochloric acid

HD - HomeoDomain

HLHS - Hypoplastic Left Heart Syndrome

HOS – Holt-Oram Syndrome

HSF - Human Splicing Finder

ISE - Intronic Splicing Enhancer

ISS - Intronic Splicing Silencer

KCl – Potassium chloride

$MgCl_2$ – Magnesium chloride

NaCl – Sodium chloride

NK2-SD - NK2-Specific Domain

NLS - Nuclear Localization Signal

PA - Pulmonary Artesia

PAPVR - Partial Anomalous Pulmonary Venous Return

PBS – Phosphate Buffer Saline

PCR - Polymerase Chain Reaction

PDA - Patent Ductus Arteriosus

PHA – Phytohaemagglutinin

PolyPhen-2 - Polymorphism Phenotyping version 2

PS - Pulmonary value Stenosis

RNS – Reactive Nitrogen Species

ROS – Reactive Oxygen Species

RPMI-1640 medium - Roswell Park Memorial Institute medium

SIFT - Sorting Intolerant From Tolerant

SNP – Single Nucleotide Polymorphism

SSC – Saline Sodium Citrate

TA - Tricuspid Artesia

TAD - Transcriptional Activation Domain

TAPVC - Total Anomalous Pulmonary Venous Connection

TE - Tris-EDTA

TN - Transactivation domain

TOA - Transposition Of the great Arteries

TOF - Tetralogy Of Fallot

VSD - Ventricular Septal Defect

ZF – Zinc Finger domain

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CHAPTER-1

Introduction

Congenital anomalies (CAs) affect approximately 1 in 33 infants and are one of the most common causes of disability in developed and developing countries. An estimated over 300,000 newborns die during the first 28 days of life every year from congenital anomalies as per the WHO report (1). In the United States, 3-5% of newborns are reported to have major congenital malformations and these account for more than a quarter of a million affected children each year (2). In India, CAs account for 8-15% of perinatal deaths and 13-16% of neonatal deaths. Genetic factors (30-40%) and environmental factors (5-10%) have been shown to be responsible for the etiology of congenital anomalies, and about 50% have been attributed to unknown causes. Among genetic etiology, chromosomal abnormalities constitute 6%, single gene disorders constitute 25% while multifactorial causes constitute 20-30% of the cases (3-5).

CAs are defined as structural or functional anomalies that occur during intrauterine life and are identified prenatally or at birth or during the first few weeks of life. It may involve a single organ or multiple organs of the body (1). CAs are classified into major and minor anomalies and the major anomalies include anencephaly, spina bifida, cleft lip and palate, heart defects, gastrochisis, omphalocele as well as several other (6). The minor anomalies include frontal bossing, epicanthal folds, hypertelorism, upslanting or downslanting palpebral fissures, flat bridge and others (6). The most common congenital anomalies are Congenital Heart Disease (CHD), neural tube defects, and Down syndrome (1).

CHDs are a group of structural abnormalities of the heart which include septal defects, valve defects and lesions that are present at birth and is considered as a complex multifactorial disorder with genetic and environmental factors playing an important role in disease development (7,8). CHD is the most common birth defect in humans affecting 1% of all live births in the first year of life and it is one of the major causes of morbidity and mortality in infants (8). In India,

nearly 180,000 children are born with CHD with a prevalence range of 8 – 10 for every 1000 live births per year (9). It is categorized mainly into four groups namely; septal defects, cyanotic heart disease, obstruction defects and hypoplasia (11).

The genetic causes of CHD include chromosomal aberrations and aneuploidies which account for about 8-10% of cases (12). CHDs also occur in about 40% to 50% of Down syndrome, Turner syndrome, Patau syndrome and Edwards syndrome patients (13,14). In addition, CHDs are prominent clinical features in a variety of syndromes caused by chromosomal abnormalities like DiGeorge syndrome (deletion 22q11.2) and Williams-Beuren syndrome (deletion 7q11.23) (15, 16). Furthermore, copy number variations have also been identified in patients with isolated (non-syndromic) heart malformations like septal defects, endocardial cushion defect, left-sided congenital heart disease, tetralogy of fallot and others (17-19).

Cardiac development is a complex process controlled by an evolutionary conserved gene regulatory network that connects transcription factors and signaling pathways with genes for muscle growth, patterning and contractility (20). A group of highly conserved transcription factors such as *GATA4, NKX2-5, TBX5, TBX20, MYH6* and others are involved in foetal heart development and regulation (21-23). Among these, *GATA4* gene is investigated extensively and is known to cause sporadic and familial non-syndromic CHDs which include Atrial Septal Defect (ASD), Ventricular Septal Defect (VSD), Patent Ductus Arteriosus (PDA), AtrioVentricular Septal Defect (AVSD), Tetralogy Of Fallot (TOF) and Pulmonary value Stenosis (PS) (21-24).

GATA proteins (DNA binding proteins) are involved in cell differentiation, survival and proliferation of tissues that regulate gene expression. They recognize and bind to "*GATA*" consensus sequence of target genes (25). Six members have been indentified in vertebrates *GATA* family. *GATA1, GATA2,* and *GATA3* are mainly involved in hematopoietic cell expression,

whereas *GATA4, GATA5* and *GATA6* are expressed in heart, liver and gonadal tissues (26). *GATA4* (Gene Id: 2626, OMIM: 600576) is a critical transcription factor expressed in embryo and adult cardiomyocytes and it promotes cardiac morphogenesis, survival and function of the heart (27). Human *GATA4* gene maps to chromosome 8p23.1- p22 region, a hypermutable protein coding gene encodes 442 amino acids with two transcriptional activation domains (TAD1, 1 – 74 amino acids; TAD2, 130 -177 amino acids), two zinc finger domains (ZF1, 215 – 240 amino acids; ZF2, 270 – 294 amino acids) and one nuclear localization signal (NLS, 254-324 amino acids) (28,29). Mutations associated in *GATA4* gene had been reported in several cardiac diseases such as CHD, abnormal ventral folding and hypoplasia of ventricular myocardium (30,31). Till date, more than 120 mutations have been reported in the *GATA4* gene. All these mutations in *GATA4* have been well-studied and have been implicated as reasons for CHD in humans (32,33).

Similarly, *NKX2-5 / CSX1* (Gene Id: 1482, OMIM: 600584) is a cardiac specific, homeobox transcription factor and is the first known marker of myocardial progenitor cells in all species. It is a highly conserved transcription factor and is also a vital regulator of cardiac structure formation and development (34). It is a member of NK homeobox gene family located on chromosome 5q34 and encodes 324 amino acids. It consists of two coding exons with homoedomain (HD), transactivation (TN) domain and NK2-specific domain (NK2-SD) (35). It is the fifth identified gene of NK-2 family gene and hence is known as *NKX2-5* and is homologous to the Tinman found in Drosophila melanogaster (36). Schott first reported heterozygous mutations in this cardiac specific gene and proved the genetic cause for the diseased condition (35). Since then, several studies have been carried out on *NKX2-5* gene both in familial and sporadic condition of CHD in humans (37,38). Mutations in this gene are known in conditions of ASD, VSD, AV

block, TOF, Ebstein malformations and tricuspid valve abnormalities. More than 40 mutations have been reported until now (39).

Other than genetic factors as explained above, oxidative stress could be a cause for CHD affecting morphogenesis as well as consequence of CHD due to defective functional heart. A healthy intrauterine life of fetus determines the birth of a healthy newborn. The intrauterine environment could interact with the genetic make up to shape the risk of diseases either at the developmental stage or diseases later in life. Fetal hypoxia has been shown as a common complication in pregnancy. Oxidative stress in the fetal heart and vasculature underlies the mechanism through which prenatal hypoxia determine cardiovascular problem. Thus oxidative stress could be one of the factors that affect the intrauterine life and post uterine life. Further, such developmental defects result in disease condition could deteriorate the oxidative stress status and can manifest additional disease condition worsening the health status of the new born. The intrauterine life again vulnerable to exposure to radiation, toxins, chemicals and all these could have varying kinds of effects on the fetus (40).

Oxygen is essential for cardiac viability, function and myocardial gene expression. During hypoxia, the level of myocardial oxygen decreases and alters gene expression patterns in the heart (41). CHDs either due to septal defect or great vessel anomaly are more prone for hypoxia (42,43). Chronic hypoxia of CHD results in a down-regulation of antioxidant defenses, making cells vulnerable to oxidative damage (44).

Free radicals are reactive compounds that are produced naturally in the body by breaking a chemical bond and keep one electron either through cleavage of radicals or by redox reactions (45). Reactive oxygen species (ROS) include both free radicals and nonfree radical oxygenated molecules (46). When ROS are elevated at higher concentrations, they generate oxidative stress

that can damage lipids, proteins and DNA (46). In DNA the guanine residue is more prone to oxidation and form 8-hydroxydeoxyguanosine (8-OHdG). The measurement of the levels of this molecule has been utilized as an index of DNA damage (47,48).

It is evident from the above descriptions that congenital anomalies are a major cause of mortality and morbidity and CHDs contribute greatly towards this. The studies on the cytogenetic and molecular aspects of CA continue to be an active area of research in order to unravel the molecular aspects in greater detail. Keeping in mind the relevance of these studies in Indian context and population, this research problem had been taken up on CA with special emphasis on CHD.

CHAPTER-2

Review of Literature

2.1 Congenital anomalies

Congenital anomalies (CAs) are defined as structural or functional anomalies that occur during intrauterine life and are identified prenatally or at birth or during the first few weeks of life. It may involve a single organ or multiple organs of the body. CAs can result in long-term disability with a significant impact on individuals, families, societies and health-care systems and are the major cause of new born deaths within four weeks of birth (1,49). CAs affects approximately 1 in 33 infants and result in approximately 3.2 million birth defect-related disabilities every year. An estimated over 300,000 newborns die during the first 28 days of life every year from congenital anomalies as per WHO report (1). According to March of Dimes report (2006), the prevalence of birth defects in India is 6-7% which converts to around 1.7 million birth defects annually. The common birth defects include CHD (8-10 per 1000 live births), congenital deafness (5.6-10 per 1000 live births), and neural tube defects (4-11.4 per 1000 live births) (49).

It is reported that in developed countries about 3% of all children born have been shown to present with significant congenital anomalies (1). Chromosomal disorders form a major category of genetic disorder accounting for a large proportion of congenital anomalies. A typical combination of anomalies affecting more than one body part is referred to as a malformation syndrome (50). CAs arises due to malformation (associated with a disorder of tissue development often occur in the first trimester), dysplasia (disorder at the organ level), deformation (arising from mechanical stress to normal tissue occurring in the second or third trimester), disruption (breakdown of normal tissues) and sequence (multiple effects occur in a specified order) or syndrome (50).

2.1.1 Classification

EUROCAT is an epidemiologic surveillance of congenital anomalies in Europe and it facilitates surveillance, clinical and epidemiological research in the field of rare genetic syndromes. According to the EUROCAT 2013 classification, it is classified into various systems with the major congenital anomalies excluding the isolated minor congenital anomalies (51). They are congenital anomalies of the nervous system, the face, the eye and the ear, the respiratory system, the cardiovascular system, musculoskeletal system, genital organs and abdominal wall defects given in the Figure 2-1 (A-H).

Courtesy:https://www.cdc.gov/ncbddd/birthdefects/surveillancemanual/photo-atlas/nervous.html (52).

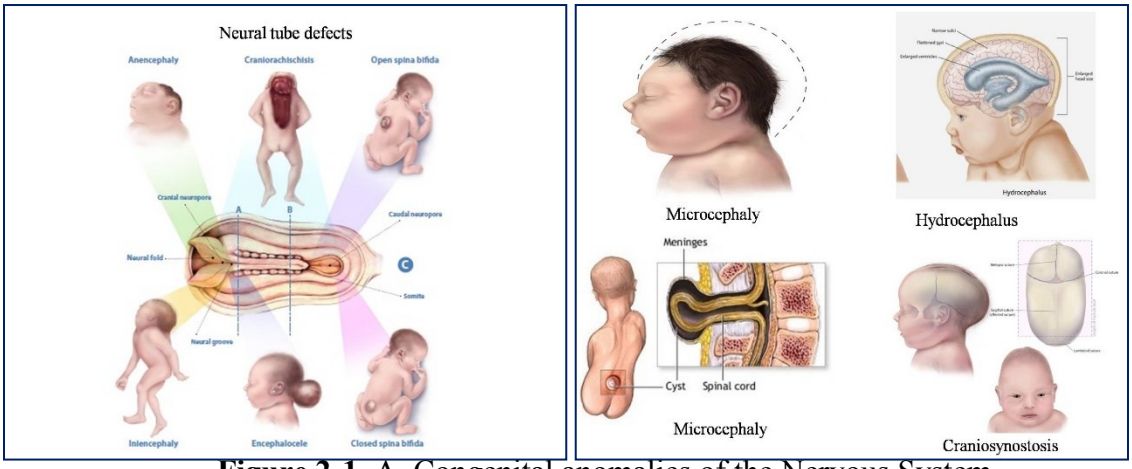


Figure 2-1. A. Congenital anomalies of the Nervous System.

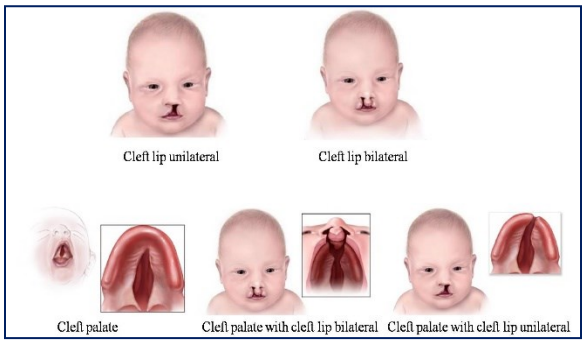


Figure 2-1. B. Congenital anomalies of the Face.

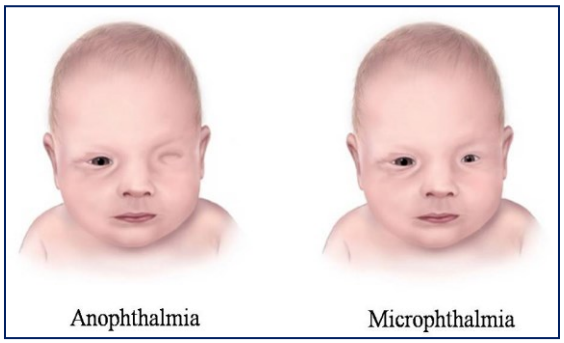


Figure 2-1. C. Congenital anomalies of the Eye.

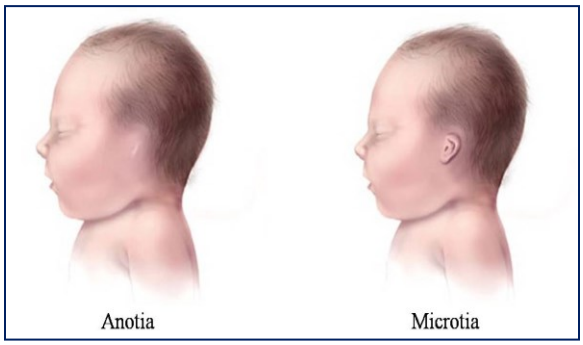


Figure 2-1. D. Congenital anomalies of the Ear.

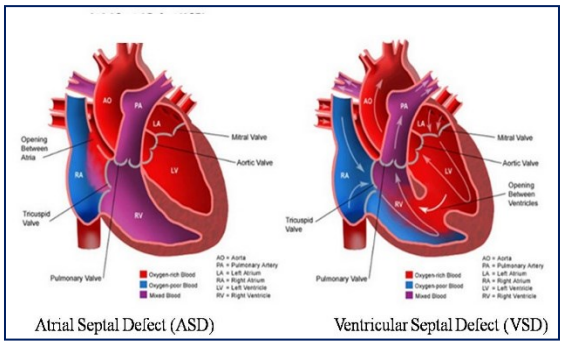


Figure 2-1. E. Congenital anomalies of the Cardiovascular System.

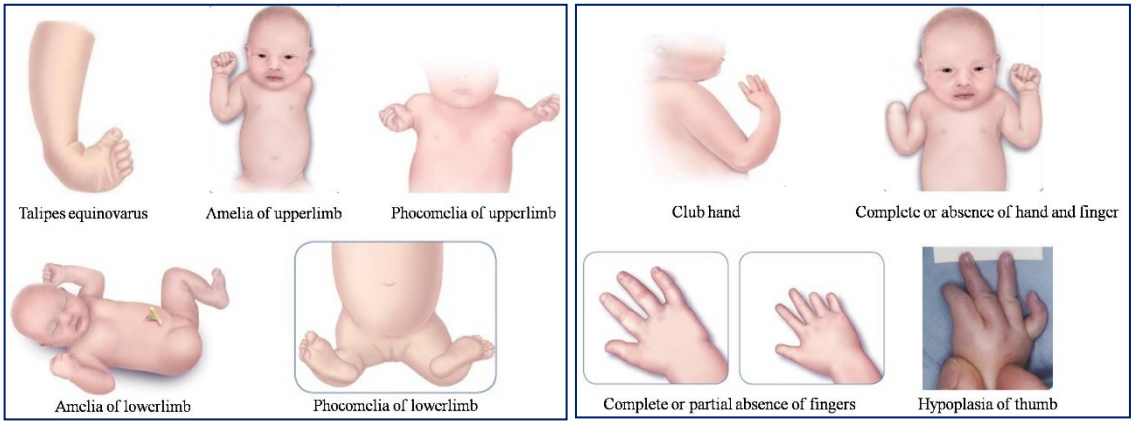


Figure 2-1. F. Congenital anomalies of the Musculoskeletal System.

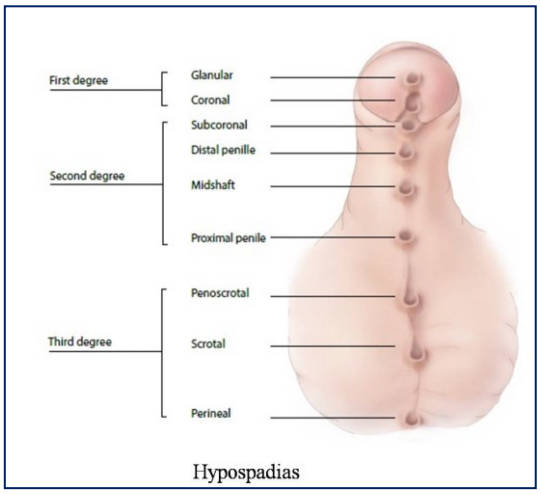


Figure 2-1. G. Congenital anomalies of the Genital organs.

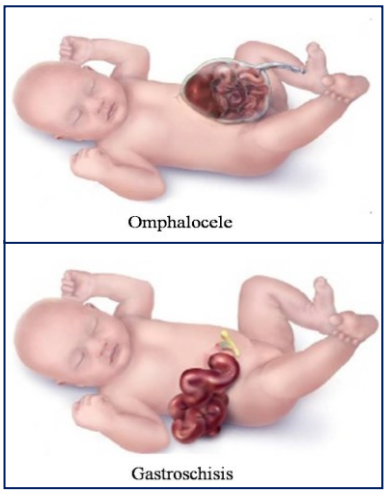


Figure 2-1. H. Congenital anomalies of the Abdominal wall defects.

2.1.2 Causes and risk factors

CAs can be caused by single gene defects, chromosomal abnormalities, multifactorial inheritance, environmental teratogens and micronutrient deficiencies (53). Maternal illnesses like diabetes mellitus, conditions such as iodine and folic acid deficiency, and exposure to medicines and recreational drugs including alcohol and tobacco, certain environmental chemicals and high doses of radiation are other factors that cause birth defects (53). The etiology of more than 50% of anomalies is still unknown even with the remarkable advances in genetic analysis over from the last decade (54). According to the Centers for Disease Control and Prevention (CDC), majority are caused by a complex mix of factors including genetics, environment and behavior (6). The etiology of congenital anomalies is genetic (30-40%) and environmental (5 to 10%). Among the genetic etiology, chromosomal abnormality constitutes 6%, single gene disorders 25% and multifactorial 20- 30% (55).

2.1.3 Chromosomal abnormalities

Chromosomal abnormalities are due to either gain or loss of chromosomal content affecting the numbers and structures of the chromosomes. Approximately 1 in 200 live newborns have been shown to have chromosomal abnormality (56). In perinatal deaths, the frequency varies between 5 - 10%, and is estimated to be more than 60% in first trimester miscarriages (57). Polyploidy and aneuploidy (monosomy and trisomy) are the two types and mosaicism which may be present in all the cells or in two / more cell lines (58). Triploidy occurs in approximately 6% of pregnancies, both polyploidy and monosomy are virtually lethal in man and the most common trisomy is Down

syndrome (trisomy 21), followed by Edwards syndrome (trisomy 18) and Patau syndrome (trisomy 13) (58).

Structural chromosome abnormalities are translocations, inversions, deletions or duplications, robertsonian translocation, isochromosomal translocation, ring formation and fragile sites (59). They may arise de novo or as a result of a parental chromosome rearrangement. Balanced carriers are entirely normal, but they are at risk of having chromosomally unbalanced offspring or miscarriages due to malsegregation at meiosis. These chromosome rearrangements also result in partial monosomy and partial trisomy (60). Microdeletion syndromes, such as Prader– Willi and Angelmann syndromes (chromosome 15), DiGeorge syndrome (chromosome 22), and Miller–Dieker syndrome (chromosome 17) are also being identified with increasing frequency (60,61).

2.1.4 National scenario:

Precise data on prevalence of chromosomal abnormalities in India is not available as many cases remain undiagnosed. Recent review by Kaur and Singh estimates the prevalence of genetic and congenital anomalies at 25-60 per 1000 births (62). A survey study conducted by Mishra and Baveja showed the incidence of congenital anomalies to be 14.64 per 1000 births. Major anomalies were seen in 1.1% and minor in 0.4% births. Multiple anomalies form the major part with an average prevalence of 37.68%. Anomalies of central nervous system and those of skin and appendages account to about 13% each (63). Prevalence of common congenital anomalies in India is shown in Table 2-1 (64).

Table 2-1. Prevalence of common congenital anomalies in India.

Malformations	Cases per 10,000
Neural tube defects	36.3
Talipes	14.5
Polydactyly	11.6
Hydrocephalus	9.5
Cleft lip and/or palate	9.3
Congenital heart disease	7.1
Hypogonadism	5.6
Trachea-oesophageal fistula	3.7
Diaphragmatic hernia	2.6
Anorectal atresia / stenosis	2.4
Microcephaly	2.2
Cleft palate	1.7
Intersex and cryptorhidism	1.6
Intestinal atresia / stenosis	1.2
Anophthalmia / microphthalmia	1.0

A prospective study of 17,653 births was undertaken in Mumbai by Patel and Adhia. The result indicated that the incidence of major anomalies was found to be 1.6% while that of minor anomalies was found to be 7.92%. The most frequent anomalies were seen in the case of central nervous system (65). Yashwanth *et al.,* conducted studies on 195 referred cases of malformation in Chennai and reported chromosomal abnormalities in 37% of the cases. All the cases belonged to known syndromes and Down syndrome was the most common syndrome (66). Jain *et al.,* analysed 101 Indian children with intellectual disability and the genetic causes were seen in 82.1% of the patients. The study found karyotype abnormalities in 15 out 33 patients and copy number variations in 1 out of 10 patients and array CGH studies on 5 cases showed abnormalities in 3 cases (67).

2.1.5 International scenario:

Congenital anomalies due to various types of chromosomal aberrations have been described extensively in the literature (4,5,68-70). Traditional method of chromosome analysis can identify abnormalities up to a limit of ~5 Mb and have limited scope to identify submicroscopic deletions and amplifications particularly in non-syndromic malformations. Array CGH as a tool for molecular genetic analysis to understand the basis of congenital anomalies is a rapidly developing trend as evidenced by a large number of reports from several countries (71). Array CGH has emerged as a robust diagnostic method for chromosomal aberrations. Diagnostic clinical genetics laboratories in Western nations are rapidly replacing conventional cytogenetic methods with array CGH as the first line test (72). The value of array CGH lies in the higher diagnostic detection rate than G-banded chromosome analysis. This is particularly important in the case of congenital anomalies of unknown etiology. The report by Ahn *et al.,* on the use of array CGH for postnatal study involved 8794 referrals ranging from neonatal congenital anomalies to adult neurodisabilities. They have reported copy number variations in 25% of the patients and out of these 87% were <5Mb which would not be detected by G-banded chromosome analysis (73). A study conducted by Uwineza *et al.,* showed copy number variations of 26% (13 out of 50 Rwandan patients) in patients with developmental delay / intellectual disability and multiple congenital anomalies. This study for the first time showed the prevalence of copy number variations in East-African population and the importance of array CGH (74). Iourov *et al.,* reported the first cohort based study on 54 Russian children affected by intellectual disability, autism and congenital anomalies with array CGH. Chromosomal imbalances were found in 48% of the cases of which 4% were novel aberrations (75). Dorfman *et al.,* analysed 35 Brazilian neonates with congenital anomalies by array CGH. The samples included in the cohort were normal by conventional

cytogenetic testing at 500-550 band level resolution. Genomic imbalances were seen in 13 (34%) of the cases (76). Serra-Juhe *et al.,* studied a cohort of 95 fetal samples with non-syndromic congenital malformations by array CGH and Single Nucleotide Polymorphism (SNP) array. Copy number variation was seen in 21% of the cases (77). The above listed representative studies showed that array CGH holds promise in discovering novel chromosomal anomalies in congenital malformations.

The advent of chromosomal microarray technique has facilitated the identification of an increasing number of submicroscopic chromosomal deletions and duplications which have been associated with a variety of congenital anomalies. Inherent duplications of some regions of the chromosomes involving strong candidate genes could present with multiple congenital anomalies (78). The reviews narrated provide some aspects of the congenital abnormalities and the utility of array CGH analysis in identifying the underlying genetic causes.

2.2 Congenital Heart Diseases (CHD)

Congenital heart disease (CHD) occurs when there is a malformation in a structural component of the heart responsible for pumping oxygenated blood throughout the body (79). CHD is the most common type of birth defect and is the leading cause of death in the first year of life (8). CHD affects 12 per 1000 live births globally and almost one third of all major congenital anomalies of heart have been observed in approximately 30% of the miscarriages (8,80).

2.2.1 Functional anatomy of the Heart

The heart is the first functioning organ to form in vertebrates required to supply the body with oxygen rich blood (81). The right side of the heart, including the right atrium, ventricle and pulmonary artery, form the pulmonary circuit with the lungs to allow for blood oxygenation. While the left side of the heart, including the left atrium, ventricle, and aorta, is responsible for sending

the oxygen rich blood systemically, throughout the body. The walls of the heart are made up of three tissue layers: myocardium, endocardium, and epicardium. The heart relies upon its four valves to ensure unidirectional blood flow. The atrioventricular (AV) valves are located between the atrium and ventricle, and include the tricuspid and mitral valves, positioned on the right and left sides of the heart respectively. The AV valves open to allow blood to flow into the ventricle and close to prevent blood from flowing back into the atria. The semilunar valves are part of the outflow tract and include the pulmonary and aortic valve, located on the right and left sides of the heart respectively. The semilunar valves ensure unidirectional blood flow through the great arteries as the blood pumps out of the ventricles. Finally, the heart muscle is supplied blood through the coronary arteries, which branch off the aorta and supply oxygen rich blood directly to the muscle of the heart described in Figure 2-2. The development of this highly organized system requires harmonious interaction between several cell lineages and molecular pathways (82).

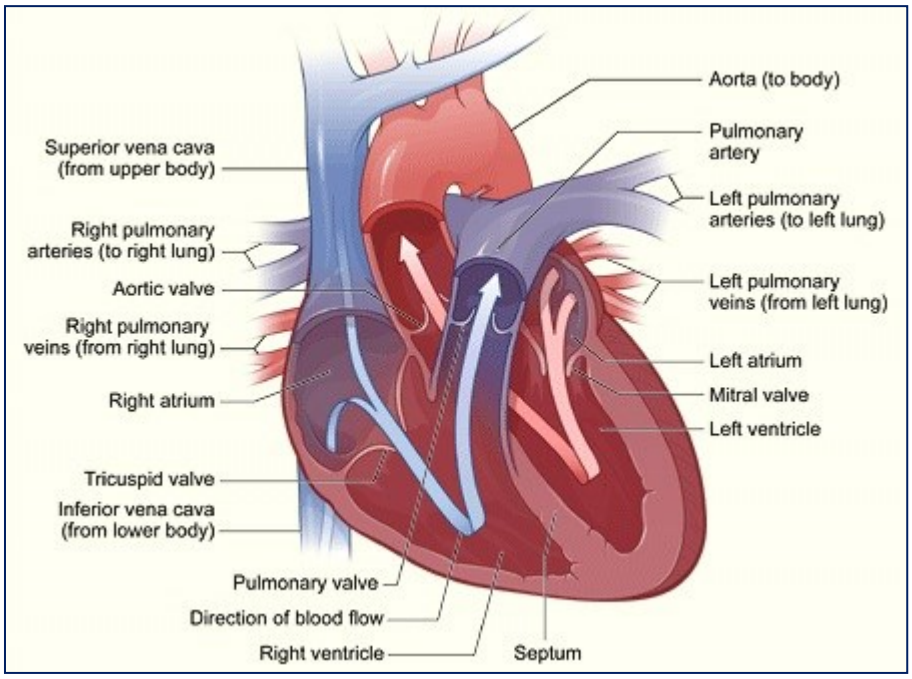


Figure 2-2. Cross-section of a healthy heart and its inside structures. The blue arrow shows the direction in which oxygen-poor blood flows through the heart to the lungs. The red arrow shows the direction in which oxygen-rich blood flows from the lungs into the heart and then out to the body.

Courtesy: http://www.nhlbi.nih.gov/health/dci/Diseases/hhw/hhw_anatomy.html

2.2.2 Types and classification of CHDs

CHDs range in type, severity and incidence and they can manifest themselves in the setting of a syndrome or isolated defect. They are classified into acyanotic and cyanotic heart defects. CHDs could affect in any part of the heart like atrial, ventricular or vascular. They are divided into three main categories, namely septation defects, cyanotic heart disease and left-sided obstruction defects. Septation defects can affect the atria (ASD), the ventricles (VSD) or structures in the central part of the heart (AVSD) (12). The overall classification of CHDs was given in the Figure 2-3.

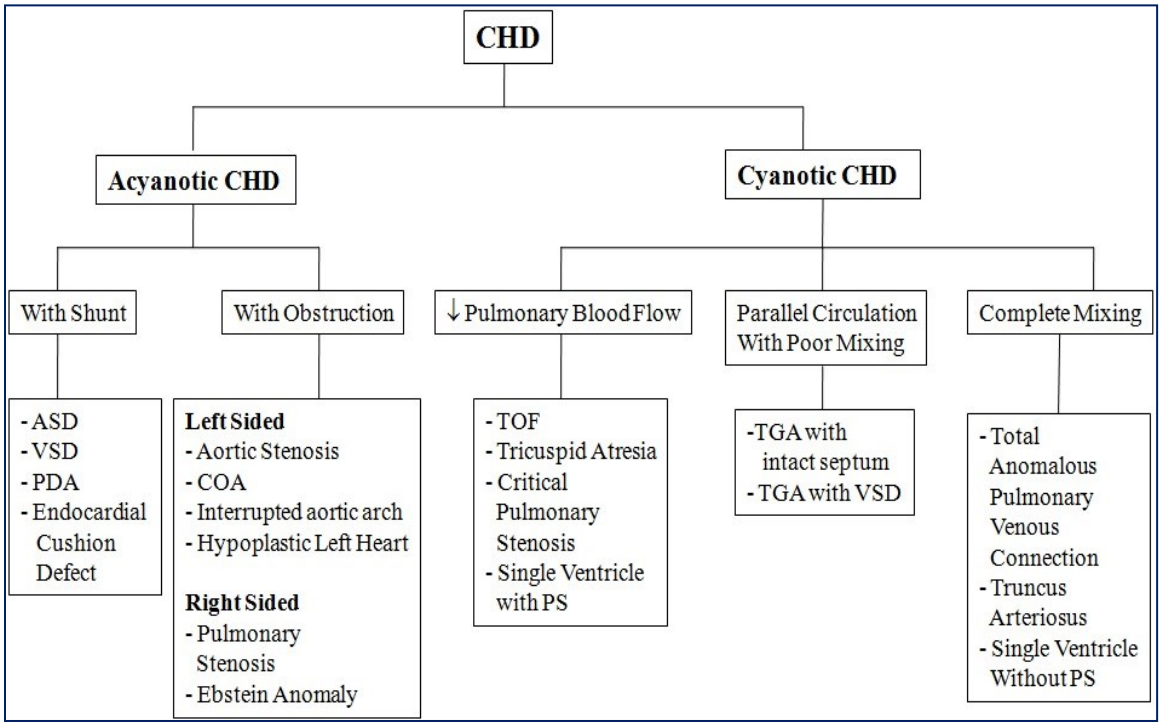


Figure 2-3. Classification of Congenital Heart Diseases

The common lesions of CHDs account for 85% of all cases which include ASD, VSD, PDA, PS, TOF, Aortic Stenosis (AS), Coarctation Of the aorta (COA), Transposition Of the great Arteries (TOA), followed by (15%) AVSD, Persistent truncus arteriosus, Tricuspid Artesia (TA), Pulmonary Artesia (PA), Total Anomalous Pulmonary Venous Connection (TAPVC), Hypoplastic Left Heart Syndrome (HLHS), Double Outlet Right Ventricle (DORV), Single ventricle / univentricular heart, Ebsteins Anomaly (EA) and Dextrocardia (heart on the right) (83). The major cardiac defects frequently reported are depicted in the Figure 2-4.

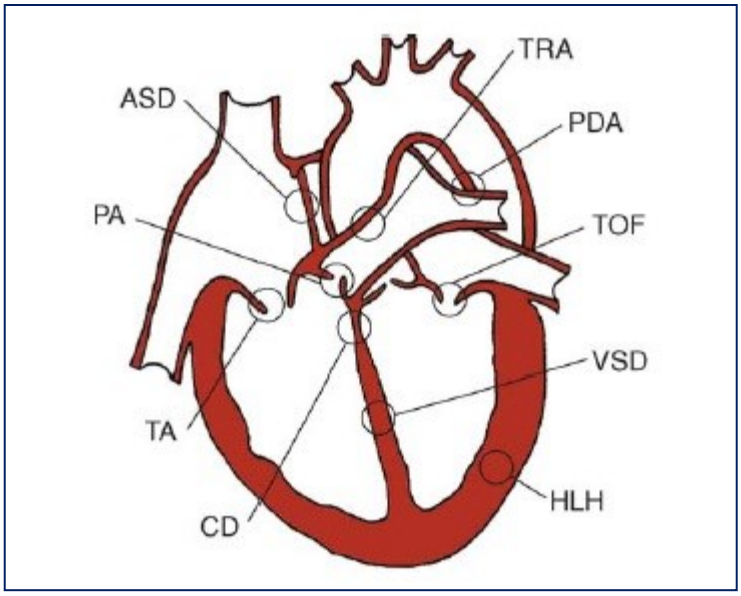


Figure 2-4. Schematic diagram shows the relative position of each cardiac defect.

Courtesy: Mandel EM, Callis TE, Wang DZ, Conlon FL. Transcriptional mechanisms of congenital heart disease. Drug Discovery Today: Disease Mechanisms. 2005; *2*(1): 33-38.

2.2.3 Incidence of CHDs

CHDs are the most common group of congenital abnormalities accounting to 30% of the chromosomal abnormalities (84). In most patients, CHDs occur as an isolated malformation, along with 33% with other associated anomalies (85). Worldwide CHDs in children continues to be a major public problem with the incidence in different studies varying from 1-17.5/1000 live births and 10% of spontaneously aborted fetus (8). The available data on CHDs in India shows an incidence of 1-5/1000 live births (86,87). The reported incidence of CHD varies substantially between different regions of the world with the highest rate in Asia (0.93%) and slightly lower rates in Europe (0.82%) and lowest in North America (0.69%). These differences might be attributed to genetic, environmental as well as socioeconomic factors (12,88).

2.2.4 Etiology of CHDs

Most of the congenital heart defects are sporadic and the major genetic cause for CHDs includes chromosomal disorders and single gene disorders (8%), environmental teratogens (2%) and 90% multifactorial disorders due to genetic and environmental factors interacting together with the development of heart (7). Increased incidence of CHDs has been noted with intrauterine viral infections, maternal drug and alcohol consumption during first trimester of pregnancy and pregnancy-induced systemic maternal disease (89). Though, most CHDs occur as a sporadic event, many diseases have been shown to have well-defined genetic basis (90). The genetic and environmental factors involved in etiology of CHDs is depicted in Figure 2-5.

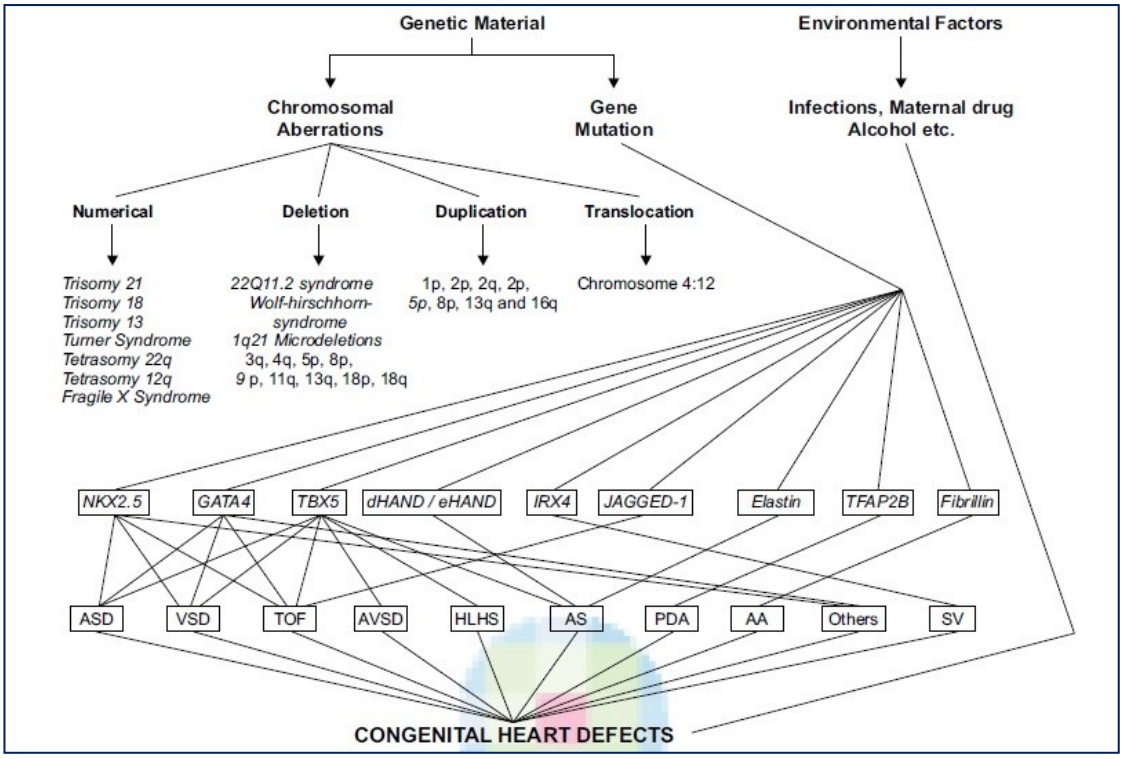


Figure 2-5. Etiology of Congenital Heart Diseases.

Courtesy: Ramegowda S, Ramachandra NB. An understanding the genetic basis of congenital heart disease. Indian J Human Genet 2005; 11(1): 14-23.

2.2.5 Chromosomal anomalies associated with CHD

The association of CHDs with chromosomal anomalies varies between 4-12% (91). They are trisomy 21, trisomy 18, trisomy 13, Turner syndrome, Tetrasomy 22q (cat eye syndrome), Tetrasomy 12q (pallister killian syndrome) and Fragile–X Syndrome. Also there are chromosome deletion and duplication syndromes associated with CHD that includes 3q, 4q, 5p, 8p, 9p, 11q, 13q, 18p, and 18q deletion syndromes. There are an equal number of duplication syndromes that also can be present with multiple congenital malformation and cardiac lesions such as 1p, 2p, 2q,

5p, 8p, 13q and 16q duplication syndromes given in the Figure 2-6. Many affected children have a combination of deletions and duplications involving the respective chromosome segments that were involved in the rearrangement. They were Deletion 22Q11.2 syndrome, Wolf-hirschhorn syndrome, 1q21 Microdeletions and other major cardiac syndromes are Noonan syndrome, Kabuki syndrome and Ellis- van Creveld syndrome (92). The aneuploidy and microdeletions and single gene defects (92) associated with CHDs are detailed in the Table 2-2 A & B.

Table 2-2. A. Aneuploidy and microdeletions associated with CHDs.

Syndromes	Cardiac Anomalies	Other Clinical Features	% with CHD
Trisomy 13	ASD, VSD, PDA, HLHS	Microcephaly, holoprosencephaly, scalp defects, severe mental retardation, polydactyly, cleft lip or palate, genitourinary abnormalities, omphalocele, microphthalmia	80
Trisomy 18	ASD, VSD, PDA, TOF, DORV,	Polyhydramnios, rocker-bottom feet, hypertonia, biliary atresia, severe mental retardation, diaphragmatic hernia, omphalocele	90-100
Trisomy 21 (Down Syndrome)	ASD, VSD, AVSD, TOF	Mental retardation, diaphragmatic hernia, omphalocele	40-50
Monosomy X (Turner Syndrome)	CoA, BAV, AS, HLHS	Short stature, shield chest with widely spaced nipples, webbedneck, lymphedema, primary amenorrhea	25-35
47, XXY (Klinefelter Syndrome)	PDA, ASD, mitral valve prolapse	Tall stature, hypoplastic testes, delayed puberty, variable developmental delay	50
22q11.2 deletion	IAA Type B, aortic arch anomalies,	Thymic and parathyroid hypoplasia, immunodeficiency, low-set ears, hypocalcemia,	75

(DiGeorge Syndrome)	truncus arteriosus, TOF	speech and learning disorders, renal anomalies	
7q11.23 deletion (Williams-Beuren Syndroome)	Supravalvar AS, PPS	Infantile hypercalcemia, elfin facies, social personality, developmental delay, joint contractures, hearing loss	50-85

Table 2-2. B. Gene defects associated with CHDs.

Syndrome	Cardiac Anomalies	Other Clinical Features	Causative Gene(s)
Noonan Syndrome	PS with dysplastic pulmonaryvalve, AVSD, HCM, CoA	Short stature, webbed neck, shield chest, developmental delay,cryptorchidism, abnormal facies	PTPN11, KRAS, RAF1, SOS1
Costello Syndrome	PS, HCM, cardiac conduction abnormalities	Short stature, developmental delay, coarse facies, nasolabial papillomata, increased risk of solid organ carcinoma	HRAS
LEOPARD Syndrome	PS and cardiac conduction abnormalities	Lentigines, hypertelorism, abnormal genitalia, growth retardation, sensorineural deafness	PTPN11, RAF1
Alagille Syndrome	PS, TOF, ASD, peripheral pulmonary stenosis	Bile duct paucity, cholestasis, typical facies, butterfly vertebrae, ocular anomalies, growth delay, hearing loss, horseshoe kidney	JAG1, NOTCH2
Marfan Syndrome	Aortic root dilatation and dissection, mitral valve prolapse	Tall stature, arachnodactyly, pectus abnormality, scoliosis, ectopia lentis, spontaneous pneumothorax, striae, dural ectasia	FBLN, TGFBR1, TGFBR2
Holt-Oram Syndrome	ASD, VSD, AVSD, progressive AV conduction system disease	Preaxial radial ray malformations (thumb abnormalities, radial dysplasia)	TBX5

Heterotaxy Syndrome	DILV, DORV, TGA, AVSD	Intestinal malrotation	ZIC3, CFC1
Char Syndrome	PDA	Dysmorphic facies and digit anomalies	TFAP2b
CHARGE Syndrome	ASD, VSD, valve defects	Coloboma, choanal atresia, developmental delay, genital and/or urinary anomalies	CHD7, SEMA3E

2.2.6 Factors promoting Cardiogenesis

The majority of the CHD genes identified are cardiac transcription factors, including GATA factors, homeobox transcription factors, T-Box transcription factors and others as depicted in Figure 2-6. Along with transcription factors, genes that play key roles in signal transduction and the formation of structural components of the heart are also identified and they include genes important in Nodal and NOTCH signaling (93).

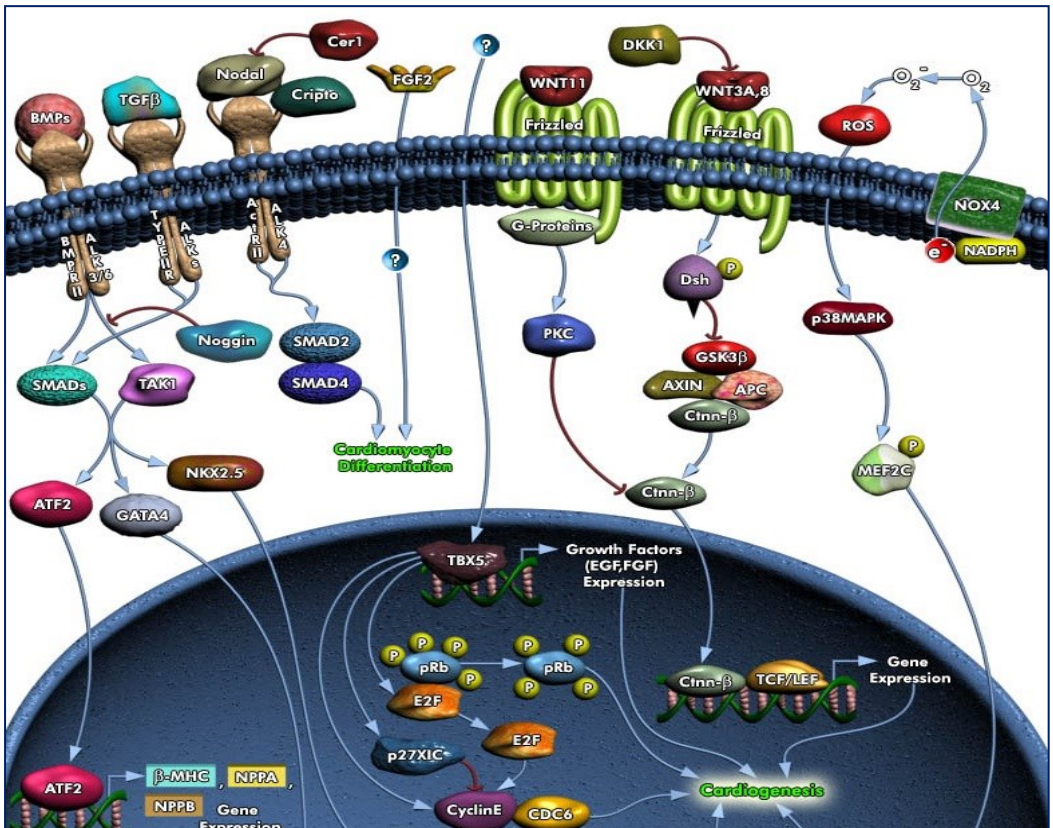


Figure 2-6. Factors promoting Cardiogenesis in vertebrates.

Courtesy: https://www.qiagen.com/es/shop/genes-and-pathways/pathway-details.

A core set of conserved transcription factors (*MEF2, TBX, NK2, GATA* and *HAND*) regulates heart development and their expression by stabilizing these cardiac gene series (94). *GATA4*, *TBX5*, and *NKX2-5* are three transcription factors that play critical roles in the cardiogenesis and these proteins interact with each another and mutations present in these genes are associated with overlapping CHD phenotypes. Garg *et al.,* identified novel mutations in non-syndromic ASD and VSD without conduction disturbances in a large pedigree family (95). Mutations in *TBX5,* which are also associated with septal defects, have been associated with the loss of interaction between *TBX5* and *GATA4* (96). *TBX5* was the first gene identified and it is associated with CHD, as mutations in *TBX5* were found to underlie the majority of Holt-Oram Syndrome (HOS) cases (97). *NKX2-5* gene mutations are also associated with septal defects, and there are studies showing that *NKX2-5* physically interacts with *TBX5* and *GATA4* (98). Mutations in *NKX2-5* gene were the first to be associated with isolated cases of familial CHD (35).

Through familial genetic studies (*in vitro* and *in vivo* analyses) identified these transcription factors as dosage sensitive and key regulators of CHD development in addition to signaling defects. The NOTCH signaling pathway is a major example of how defective molecular signaling can lead to CHD. Garg *et al.,* identified a novel mutation in *NOTCH1* which was associated with a family with outflow tract defects, including bicuspid aortic valve, aortic valve calcification and outflow tract defects (100). Furthermore, in a whole exome sequencing screening study performed by Preuss *et al.,* in 2016, additional variants were identified in *NOTCH1* in familial cases of left ventricular outflow tract defects (101). Finally, a recent study in 2017, found that a patient with HLHS had an inherited mutation associated with BAV on his maternal side and

an additional NOTCH1 mutation from his paternal side. Through the utilization of iPSCs and conversion to cardiomyocytes, it was found that NOTCH signaling was significantly dysregulated, as was cardiogenesis due to impaired nitric oxide signaling (102). Several genes have also been identified as having an involvement to these septal defects. Familial and population based studies have identified mutations in *CRELD1, ALK2, BMP2*, and *ZIC3* associated with atrioventricular septal defects (103,104). In addition, structural genes such as *MYH6* and *ACTC1* have been associated with ASD (105,106) and mutations in *TLL1* and *GATA6* also have been found to be associated with ASD (107).

2.2.7. Gene mutations

Till now more than 40 cardiac specific candidate genes have been identified which cause CHDs in human viz., *GATA4, NKX2.5/CSX, TBX5, TBX20, MYH6* and others (21-23).

2.2.7.1 GATA4

Human *GATA4* gene spans 50kbp and maps to chromosome 8p23.1- p22 region which recognizes GATA motif presented in several gene promoters. It is a hypermutable protein coding gene (108), with 442 amino acids comprising seven exons (first exon is non-coding, other six were coding exons) (109), with two transcriptional activation domains (TAD1, 1 – 74 amino acids; TAD2, 130 -177 amino acids), two zinc finger domains (ZF1, 215 – 240 amino acids; ZF2, 270 – 294 amino acids) and one nuclear localization signal (NLS, 254-324 amino acids) detailed in Figure 2-7 (110). TADs are important for *GATA4* transcriptional activity and ZF1 is essential for DNA sequence recognition and binding to consensus motif, ZF2 is involved in sequence specificity and stability of protein-DNA binding and NLS region is associated with subcellular trafficking and *GATA4* nuclear distribution (111). In addition, there are reports in support of *GATA4* as an upstream regulator of number of genes expressed during embryogenesis and cardio

morphogenesis which encode for atrial natriuretic factor, brain natriuretic peptide, α and β myosin heavy chain, vascular endothelial growth factor and cardiac troponin C and I (112,113).

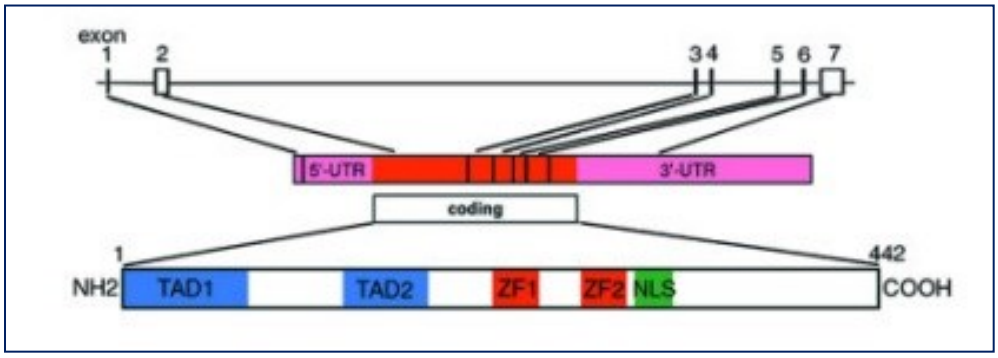


Figure 2-7. Schematic representation of GATA4 (genomic, mRNA and protein level). Courtesy: Reamon-Buettner SM, Cho SH, Borlak J. Mutations in the 3'-untranslated region of GATA4 as molecular hotspots for congenital heart disease (CHD). BMC Med Genet. 2007; 8: 38.

Studies from different countries proved the association between cardiac septal defects and *GATA4* mutations, whereas in India there are very few reports to prove the genotype-phenotype correlation among CHD patients (114-116,122). Mattapally *et al.,* identified a novel promoter mutation (c.620C>T), one splice junction mutation and one intronic mutation in *GATA4* gene associated with ASD, TOF and VSD in South Indian patients. Their studies have proved that the genetic variation (rs73203482) is pathogenic which affect the binding of splicing factor, SFR26 involved in alternative splicing site selection. They also have reported p.P394T and p.D425N mutations in Dravidian population (116). However, other two reports from India studied only the known *GATA4* gene variants and found the association with CHD (114-116).

Earlier studies indicated that *GATA4* may contribute to the congenital heart disease due to disruption of the *GATA4* gene either by a deletion or duplication at 8p23.1 region (117). Garg *et al.,* in 2003 first described about a heterozygous missense mutation (p.G296S) and a frame shift

mutation (c.1075delG) in 2 unrelated large families with ASD. They also found the interaction of *GATA4* with *TBX5* mutations to cause cardiac septal defects (118). Okubo *et al.,* identified novel c.1074delC mutation in a large Japanese family with ASD (119). Followed by them, Hirayama-Yamada *et al.,* investigated 16 unrelated families with ASD and found a novel mutation (p.S52F) and a known mutation (c.1075delG) with 12.5% prevalence (120). Later on, Sarkozy *et al.,* also identified p.G296S mutation in 29 ASD Italian populations (121). This variant (p.G296S) was not picked up in Indian population conducted by Ramegowda et al. confirming that it can be population specific (122).

Tomita-Mitchell *et al.,* identified 4 missense variants (Gly93Ala, Gln316Glu, Ala411Val, Asp425Asn) in a large population of 628 unrelated patients with 1.6% prevalence of ASD and 1.5% of VSD condition (123). Chen *et al.,* found a novel p.K300T mutation in a large family with ASD condition (124) and Xiang *et al.,* investigated a family of three generations with ASD and PS condition and found a novel p.K319E mutation (125). There are many studies reporting novel variants and CHD prevalence from different population such as Chinese 2.1% (126), Australian 1.4% (127), Indonesian <1% (128), Japanese 0.9% (129) and American 0.8% (123). Data available from Human Gene Mutation Database (HGMD; www.hgmd.cf.ac.uk), there is an extensive record of 123 non-synonymous mutations with various types of CHDs such as ASD, VSD, TOF, PS, PDA, AVSD, CoA and so on (124).

GATA4 is expressed in adult vertebrate heart, gut epithelium, and gonads. During fetal development, *GATA4* is expressed in yolk sac endoderm and cells involved in heart formation (130). Human heart *GATA4* cDNA was first cloned and identified that it may regulate a set of cardiac-specific genes which plays a crucial role in cardiogenesis. Promoter and enhancer studies suggested that this factor may regulate genes critical for myocardial differentiation and function

(131,132). Molkentin e*t al.*, reported that *GATA4* regulates the expression of *MYH6*. They identified a GATA motif located within the proximal promoter region of the *MYH6* gene (133). Durocher et al. demonstrated that *GATA4* and *NKX2.5* specifically cooperate in activating atrial natriuretic factor and other cardiac promoters, and physically interact both in vitro and in vivo (132).

Studies from animal experiments (transgenic mice) also substantiated the association of GATA4 mutants with cardiac abnormalities of septal defects, tetralogy of fallot, cardiomyopathy, endocardial cushion defect, double outlets of right ventricle and right ventricular hypoplasia which is identical to human diseased condition (134). Over expression of a missense mutation (p.V217G) in a highly conserved zinc finger domain leads to the embryo death with cardiovascular developmental deformities (135). Another study stated the increased susceptibility of GATA4 association to VSD in the embryonic hearts of knock-down chicks, bilateral myocardial rudiments failed to move to the midline forming cardia bifida anomaly (136). Rajagopal *et al.,* studied mice heterozygous *GATA4* mutation that resulted in GATA4 protein level reduction, and observed various CHD conditions (137). Qian and Bodmer stated that *NKX2-5* and T-box factors play an important roles in establishing and maintaining heart function additionally and partially through another key regulator, *TBX20* (138).

Intragenic *GATA4* mutations can cause isolated CHDs, septal defects, but PS, TOF and other defects have also been reported (118,123,137). Moskowitz IP *et al.,* demonstrated that human missense mutations in *GATA4* were shown to disrupt GATA4–SMAD4 interactions in the BMP/TGF-β signaling pathway, likely causing AVSD and valve abnormalities in the affected patients (139). Embryonic development in *GATA4* deficient mice is arrested at E10.5 with incorrect ventral folding, endodermal malfunctions and causing an inability to establish a primitive

heart tube (140). Furthermore, it has also been shown that mice heterozygous for *GATA4* mutations develop septation and endocardial cushion defects (137). *GATA4* G296S mutation have been associated with ASDs and PS in multiple human families and *in vitro* studies suggested that *GATA4* G296S mutant protein resulted in specific functional deficits including decreased DNA binding affinity, transcriptional activity and loss of a protein-protein interaction with *TBX5* (141). Direct downstream targets of *GATA4* include *HAND2* and *MEF2C* required for second heart field development (142,143). Additionally, *GATA4* and *TBX5* double heterozygous mice develop cardiovascular defects, which point towards a genetic interaction (144). All these studies reveal that *GATA4* is a crucial disease gene, as it plays a vital role in normal function and development of the heart.

2.2.7.2 NKX2-5

NKX2-5 is one of the transcription factors involved in the cardiac morphogenesis and development and it binds to the 5'-CAAGTG-3' motif in target promoters (145). It is organized with 3.125 Kb with a transcript length of 1.580 bp and is well known for its hyper-mutability potential (146). Structural organization of *NKX2-5* gene is illustrated in the Figure 2-8. Studies have shown that it plays an important role for cardiac progenitor determination, cardiomyocytes differentiation, cardiac morphogenesis and conduction system in the embryonic heart. It is also required for cardiomyocyte homeostasis and postnatal formation of ventricular conduction system in adult heart (147). *NKX2-5* weakly interacts through the homeodomain physically and synergistically with *TBX5* and *GATA4* gene to form a complex leading to CHD (148).

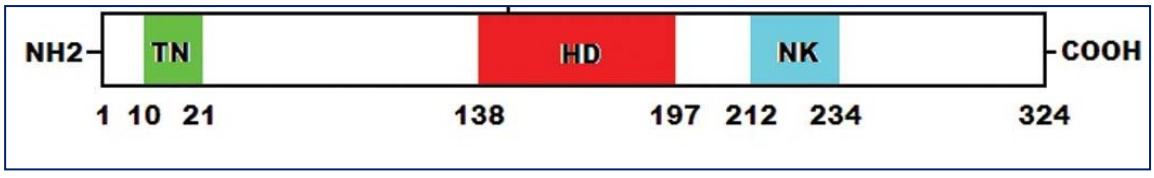


Figure 2-8. Structure of *NKX2-5* gene.

Courtesy: Yuan F, Qiu XB, Li RG, Qu XK, Wang J, Xu YJ, et al. A novel NKX2-5 loss-of-function mutation predisposes to familial dilated cardiomyopathy and arrhythmias. Int J Mol Med. 2015; 35(2): 478-86.

Data from several studies identified both germline and sporadic *NKX2-5* mutations responsible for the CHD malformation. It has been frequently reported in homeodomain region followed by TN domain and NK2-SD domain and also one in splice-site junction. Mutations associated with HD and NK2-SD domain lead to truncated protein and thus these regions plays an essential role for the construction of the conduction system (149). Mutations in HD domain particularly lead to either loss or reduced DNA binding, transactivation activities and protein-protein interactions (150).

Studies from different countries proved the association between cardiac septal defects and *NKX2-5* gene mutations, whereas in India there are very few reports to prove the genotype-phenotype correlation among CHD patients (151-152). However, these studies were on the *NKX2-5* sequence variants and attempting to find new sequence variants as well as the association between the known sequence variants with respective CHD condition. Ketharnathan *et al.,* reported the absence of *NKX2-5* mutation in the study group and suggested that exon 1 region might not be involved in CHD condition and it could be mosaic in nature. It was also proposed that further extensive analysis necessary for tissue-specific mutations (152). Dinesh *et al.,* found that the common SNPs (c.239A>G) were seen equal in both cases and control group and due to this neutral effect of synonymous SNP (Glu21Glu) this might not involve in the disease manifestation. They also identified 1212G>T SNP in 3'UTR region in 40% of CHD cases (151).

In humans, disease causing mutations in the *NKX2-5* gene HD region result in various CHDs including ASD, VSD, TOF and DORV (153-155). Septal defects and atrio-ventricular conduction defects are commonly seen in patients with a mutated *NKX2-5* gene (154). *NKX2-5* functionality is crucial in mice as homozygous mutations cause embryonic lethality due to faulty cardiac looping and insufficient myocardial differentiation during chamber formation (156). Studies on mouse revealed that *NKX2-5* gene dosage is critically important for proper regulation and cardiac conduction system as *NKX2-5* null mice lack the primordium of the AV node and the conduction system of heterozygous mutant embryos only contain half the normal number of cells (157). Additionally, Pashmforoush *et al.,* demonstrated that ventricular-restricted *NKX2-5* knockout mice displayed progressive complete heart block and massive trabecular muscle overgrowth. *NKX2-5* ranks high in the cardiac regulatory hierarchy and is expressed in both the first and second heart field (158). Furthermore, it has been demonstrated that *NKX2-5* interacts with *GATA4* suggesting that the proteins cooperate in the transcriptional activation of cardiac specific genes (159,160). *NKX2-5* interacts with *TBX5* and these two factors were shown to activate a cardiac-specific Nppa promoter (in vitro) in a synergistic fashion and also in the development of the cardiac conduction system in vivo (161).

2.2.7.3 TBX5

T-box transcription factor TBX5, is a protein encoded by the *TBX5* gene and it contains 9 exons and spans more than 47 kb and maps to chromosome 12q24.21 region. It acts as a transcription factor which is mainly involved in the development of forelimb and heart (162). Mutations in this gene can cause HOS or Amelia syndrome and these mutations are responsible for the inactivation and consequently affect the development of heart and upper limbs (163).

T-box (or Tbx) proteins bind specific DNA motifs, called TBEs (T-box Binding Elements), to activate or repress target promoters. *TBX5* appears to act essentially as a transcriptional activator and cooperates with other transcription factors such as *GATA4* and *NKX2.5* to synergistically regulate downstream targets. Another study stated that a molecular pathway including *ID2*, *NKX2-5*, and *TBX5* coordinates specification of ventricular myocytes into the ventricular conduction system lineage (164). Li et al. showed that the *TBX5* gene was mutated in cases of familial and sporadic HOS. Later on, many studies have been conducted to find out mutations associated with various clinical conditions (165). More than 70 mutations in the *TBX5* gene have been found and most of these mutations prevent the T-box 5 protein production. *TBX5* haplo-insufficiency in HOS causes cardiac and forelimb abnormalities and *TBX5* deficiency in homozygous mice decreased the expression of multiple genes and caused severe hypoplasia of posterior domains in the developing heart (166).

2.2.7.4 TBX20

T-box transcription factor *TBX20* (T-Box 20) is a protein coding gene and it encodes a deduced 297-amino acid protein and essentially expressed in the fetal heart, eye, and limb. Diseases associated with *TBX20* include ASD4 and PFO and it acts as a transcriptional activator and repressor required for cardiac development (167). Mutations in this gene are associated with diverse cardiac pathologies, including defects in septation, valvulogenesis and cardiomyopathy (168). Studies in mouse, human and fruit fly have shown that this gene is essential for early heart development, adult heart function and yolk sac vasculature remodeling and has been associated with congenital heart diseases (169).

TBX20 gene contains 8 exons, spans around 22 kb of genomic DNA which maps to chromosome 7p14.2 region. Tbx20a, Tbx20b, and Tbx20c interacted via their T-box with the

cardiac transcription factors *NKX2-5, GATA4* and *GATA5* to activate cardiac gene expression (170). Kirk et al. reported missense and nonsense germline mutations within the T-box DNA-binding domain of human *TBX20* gene that were associated with congenital heart disease and a complex spectrum of developmental anomalies (171).

2.2.7.5 MYH6

MYH6 (Myosin Heavy Chain 6) is a protein coding gene, primarily expressed in atrial tissue and maps to the chromosome 14q11.2 region. It encompasses 26,159 bp and consists of 39 exons. Cardiac muscle myosin is a hexamer consisting of two heavy chain subunits, two light chain subunits and two regulatory subunits (172). Mutations in *MYH6* gene are most commonly associated with ASDs, familial hypertrophic and dilated cardiomyopathies. It impairs the binding of the myosin heavy chain to its regulatory light chain. *MYH6* null chick embryos demonstrate disrupted formation of the atrial septum (173). *MYH6* expression is significantly down regulated when *TBX5* mutations physically associate with *MEF2C* to synergistically activate *MYH6* transcription. *NKX2-5* is also involved, acting as an upstream regulator of *MEF2C*. *GATA4* mutations that result in ASD decrease transactivation of *MYH6*, implicating *GATA4* as an upstream regulator (174).

2.2.8 Gene interactions pathways

Transcription factors are major regulators of developmental processes and play essential roles in cardiogenesis. *NKX2-5, GATA4,* and *TBX5* are perhaps the most and well studied cardiac transcription factors implicated in CHD patients and all three are very critical for development of the heart. The core cardiac transcription factors function in a mutually reinforcing transcriptional

network where each of the factors will regulate the other gene expression (95,123,175). It also function as biochemical partners for each other, reflecting a complex molecular and genetic interplay controlling multiple stages of heart and conduction system development. Schematic illustration of these septal defects, vessels and valve defects which are associated with gene mutations along with the transcription factors are given below in the figure 2-9 (176).

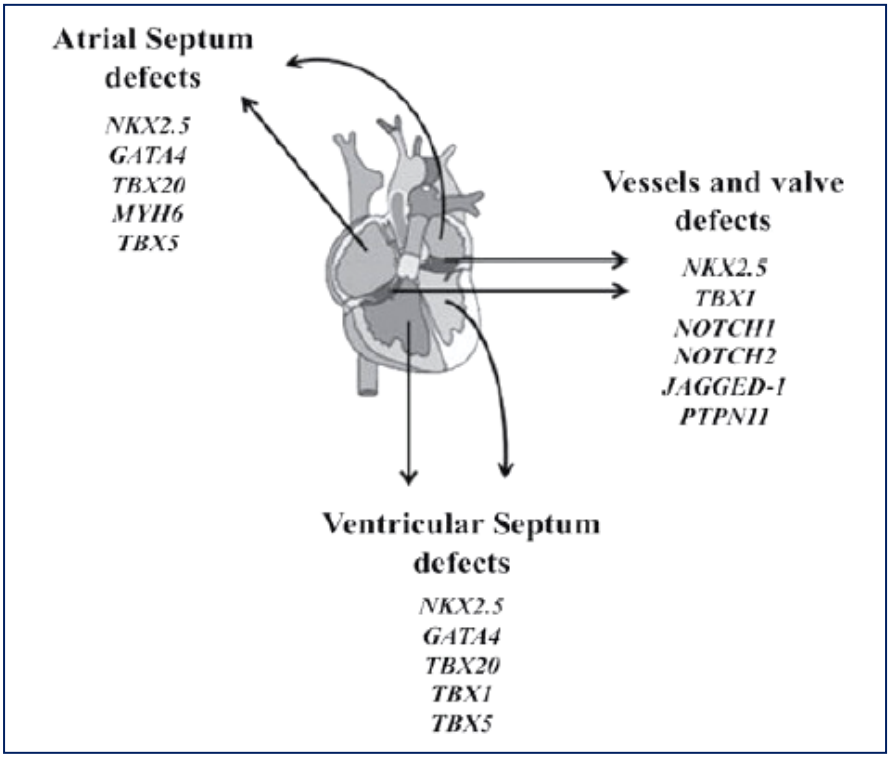


Figure 2-9. Schematic illustration of ASD, VSD and vessels and valve defects are associated with gene mutations in transcription factors and signaling molecules.

Courtesy: Vecoli C, Pulignani S, Foffa I, Andreassi MG. Congenital heart disease: the crossroads of genetics, epigenetics and environment. Curr Genomics. 2014; 15(5): 390-9.

Cardiac development is also controlled by a large number of signaling pathways, which are well regulated in the developmental networks (177). Studies on CHD genes suggest that developmental signaling pathways were involved in human CHD as ligands (e.g. *JAG1, CFC1*)

receptors (e.g. *NOTCH, PDGFRA*) down-stream signaling effectors (e.g. *PTPN11, SMAD6*) transcription factors (e.g. *GATA4, NKX2-5*) and targets (e.g. *ACTC1, MYH6*) as represented in Figure 2-10. In addition, there are other genes encoding histone-modifying proteins (e.g. *CHD7, KMT2D*) suggesting that regulation of these unknown target genes also might add additional information on regulation of cardiac developmental networks (177).

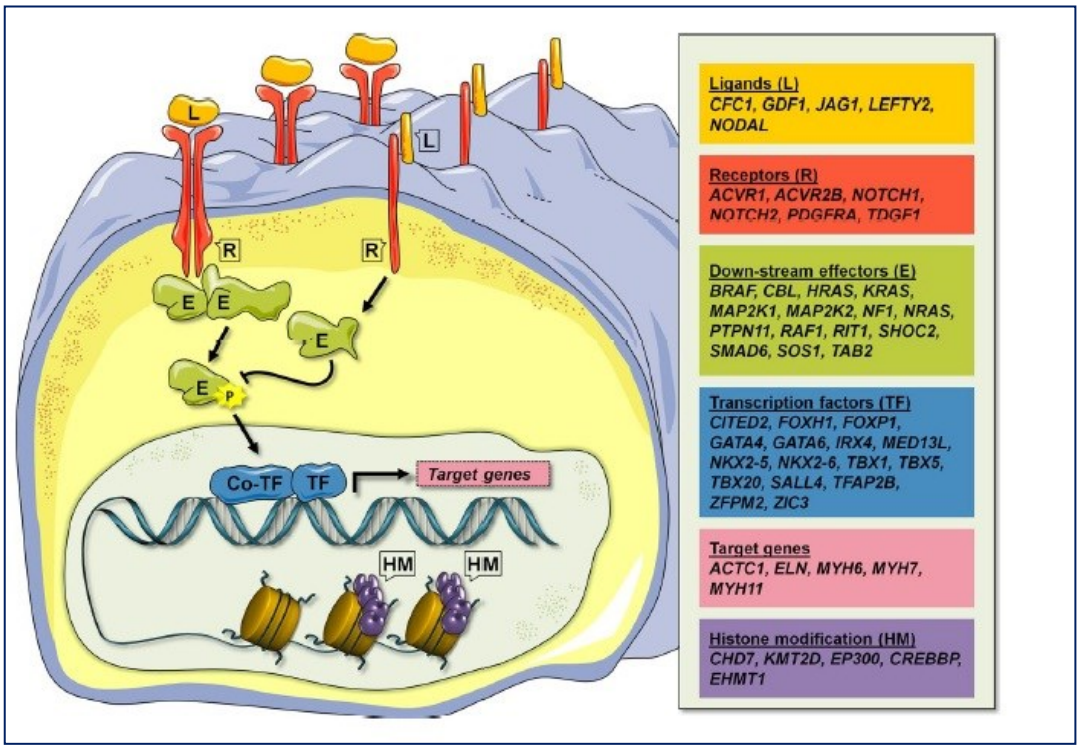


Figure 2-10. Schematic representation of the different cell signaling components affected by mutations in CHD candidate genes.

Courtesy: Andersen TA, Troelsen Kde L, Larsen LA. Of mice and men: molecular genetics of congenital heart disease. Cell Mol Life Sci. 2014; 71(8): 1327-52.

Over the past decade, substantial progress has been made in the study of the molecular genetics of the CHDs and the first genetic cause of CHD was identified in cases of syndromic CHD where chromosomal aneuploidy were associated with CHD and this includes trisomy 21, 18,

13, and Turner syndrome (178-180). Around 50% of individuals with trisomy 21 exhibit CHD phenotypes ranging from septal defects to atrioventricular canal lesions. In cases of trisomy 18 nearly all affected individuals will display septal defects. Additionally, trisomy 13 has an 80% incidence of CHD, typically in the form heterotaxy and laterality defects. Apart from that, around one third of females were with Turner syndrome (181). In addition to chromosomal abnormalities, the other genetic causes including copy number variations and point mutations were also play significant role to the genetic contribution of CHDs (181). A recent review by Samir Zaidi and Martina Brueckner provides the percentage of various genetic and environmental causes of CHD. Aneuploidy was detected to the extent of 13%, CNV 10%, known gene inherited 1%, de novo chromatin single nucleotide variant (SNV) 3%, other de novo SNV 7%, environmental 10% followed by unknown cause to the extent of 56% (182). Further, gene-environment interactions also play an important role towards the genetic causes of CHD. This also provides a major contribution to other CHD comorbidities such as heart failure, arrhythmia and neurocognitive outcomes (183). As CHD contributes to major portion towards the overall burden of cardiovascular disease (184), thorough understandings of the underlying genetics were required to improve care of CHD patients.

Studies from epigenetic mechanisms demonstrated the environmental influences on cardiogenesis and it was carried out on histone modifying enzymes and chromatin remodeling complexes in heart development of mice (185) and their role for human CHD was confirmed by showing *de novo* mutations in histone modifying genes (186). Further, changed expression of chromatin remodeling factors in CHD patients has been studied by Sheng et al. (187). In addition, DNA methylation studies of the cardiac transcription factors *NKX2.5*, *HAND1* and *TBX20* were

identified in cardiac biopsies of TOF patients and showed altered levels of methylation in CHD patients (188,189).

MicroRNAs and lncRNAs also have been shown to play important roles in cardiac development (190). Studies carried out on CHD patients showed altered expression of miR-196a2 in bicuspid aortic valve between stenotic and insufficient valves (191,192). These genetic and epigenetic factors lead to molecular network imbalances underlying heart development as demonstrated by distinct gene expression profiles characterizing different types of CHDs (192).

From the above discussed review, it is clear that transcription factors (*GATA4, NKX2-5, TBX5 etc.*) constitute the core regulatory network that is responsible for normal cardiac morphogenesis and genes involved in the development of CHDs.

2.3 Oxidative DNA damage

Oxidative stress is the state when there is a disturbance between the production of free radicals and antioxidant defenses in the human body (193). Free radicals are molecules with unpaired electron surrounding in their outer orbit. They are highly reactive and unstable and they can react with other compounds in order to gain the extra electron for their stability and resulting in cell disruption (194). Reactive oxygen species (ROS) are ubiquitous in living aerobic organisms and they result either from the cells metabolism or by exogenous physical sources (e.g., ionizing radiation, UV rays) and/or chemical compounds (195). Biological damage caused by ROS has been proposed to contribute to aging as well as a number of degenerative processes associated with aging such as cancer, heart disease, and etc. (196).

Oxygen is essential for cardiac viability, function and myocardial gene expression (197). Due to the structural defect or increased pulmonary blood flow in CHD, it develops chronic hypoxia and lead to gradual or sudden onset of cyanosis (198). CHD usually divided into two

groups cyanotic and acyanotic heart defects. In the case of non-cyanotic (acyanotic) heart defects, blood flows from the left side of heart to the right side of the heart due to structural abnormality. Individual with left to right shunting often retain or present with normal oxygenated saturation in systemic circulation causing left to right shunt lesion which include ASD, VSD, PDA , ECD and PAPVR. Left to right shunt causes an elevation of pulmonary blood flow, which triggers obstructive and obliterate alteration in the pulmonary vascular bed and a progressive increase in pulmonary vascular tissue (199-202). Congenital Heart Diseases (CHD), either due to septal defect or great vessel anomaly is more prone for hypoxia (43,203,204). Chronic hypoxia of CHD results in a down-regulation of antioxidant defenses, making cells vulnerable to oxidative damage (205).

Oxidative DNA damage in CHDs have been demonstrated by comet assay on cultured lymphocytes as well as measurement of biochemical parameters such as glutathione peroxidase, superoxide dismutase, catalase, vitamin E, uric acid, selenium and malondialdehyde (MDA) (205-207). Rokicki et al. evaluated the oxidant and antioxidant status in 23 infants suffering from congenital heart diseases (14 with left-to-right shunt and 9 with cyanotic heart defects) and 18 healthy infants. They evaluated the levels of antioxidant enzymes, low molecular weight antioxidants and malondialdehyde as a marker of lipid peroxidation. The results showed an imbalance and it was significantly higher in cyanotic heart defects than in the healthy controls (208). Ercan et al. conducted a case-controlled, cross-sectional study on 32 healthy children, 30 children with acyanotic heart disease and 29 children with cyanotic heart disease. They reported that the plasma total antioxidant status (TAS), total oxidant status (TOS) and oxidative stress index (OSI) were significantly higher in children with cyanotic heart diseases than in children with acyanotic heart diseases and healthy controls (209). Pavlova et al. also showed the induced antioxidant response and accumulation of oxidized products in CHD and TOF patients (210).

Rivera et al. measured the levels of 8-OHdG with lipid peroxidation and showed that it was higher in heart failure patients than with controls (211).

These studies have indicated significantly higher oxidative stress in cyanotic group than the acyanotic group. The anatomical defect in CHD could develop hypoxia and increase the levels of free oxygen radicals (205) leading to cellular and molecular damages. Free radicals can damage all cell components including lipids, proteins and DNA. In DNA, guanine base is more prone to oxidation (C-8 of guanine) and is one of the most common oxidative events resulting to mutagenic lesion (212). 8-OHdG is the most studied oxidative DNA lesion and is the representative compound that reflect oxidative cellular DNA damage induced by reactive oxygen species (213) and its formation was first reported in 1984 by Kasai and Nishimura (214). The measurement of the levels of this molecule has been utilized as an index of DNA damage (215).

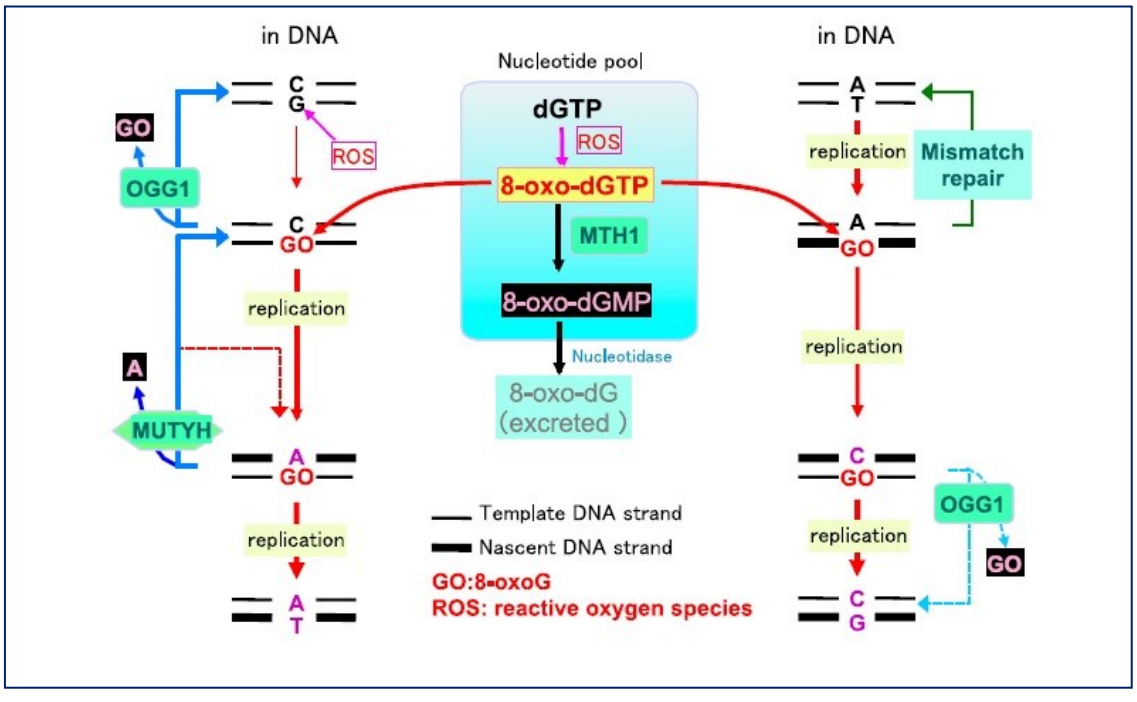


Figure 2-11: Mutagenesis caused by 8-oxoguanine.

Courtesy: Nakabeppu Y. Cellular levels of 8-oxoguanine in either DNA or the nucleotide pool play pivotal roles in carcinogenesis and survival of cancer cells. Int J Mol Sci. 2014; 15(7): 12543-12557.

The consequences of 8-OHdG base in the double stranded DNA on replication have shown to be with tremendous mutagenic potential. It can base pair with cytosine or adenosine bases resulting in G:C to T:A transversion mutations as depicted in Figure 2-11 (216). It could be deciphered from the figure that 8-OHdG could have some major effects in the replication process leading to mutational (base) changes, effects on transcriptional and finally on translation, yielding functionally ineffective proteins.

The review of literature described here clearly indicates some of the major chromosomal anomalies in congenital anomalies in general and CHDs in particular. It could be seen that in many of the reports chromosomal analysis did not yield any conclusive information and it required advanced techniques like array CGH or studies at gene level to obtain the underlying cause for the disease manifestations. Thus it is imperative that molecular studies highly pertinent to unravel the cause for the diseases. The reports on molecular analyses of the candidate genes of cardiogenesis have been studied extensively and the defects in the genes also have been consolidated. Further, CHDs are known to cause oxidative stress and damage the cellular and molecular architecture affecting primarily lipids, proteins and DNA. Information available on this aspect indicate probable mutations on account of transversion highlights its damaging role in the process of transcription and also in translation.

CHAPTER-3

Lacunae of Knowledge

There are several studies investigating the profile and pattern of congenital anomalies in various ethnic populations (3-5,62-67). The present study is aimed to systematically screen the congenital anomalies in children being treated at R. L. Jalappa Hospital & Research Centre, a tertiary care teaching hospital. The study would encompass chromosomal aberrations as well as molecular underpinnings associated with dysmorphism, developmental delay in general and CHDs in particular. Though, CHDs have been one of the commonest genetic defects in children and the genetic basis of CHDs has not been explored in depth in the Indian population except for few studies (115,151,152). Genes namely *GATA4*, and *NKX2.5* were shown to be associated with cardiac morphogenesis (95,132, 217).

Whole gene sequencing has not been done in India and there is a paucity of literature and lacuna in knowledge on the molecular underpinnings of CHD in Indian population. Therefore, it was considered worthwhile investigating the sequence variants in *GATA4* and *NKX2-5* genes in a spectrum of various CHDs. The outcome of the study is expected to provide the molecular insights into congenital heart anomalies and the sequence variants in exonic and intron-exon boundary regions. In addition, a systematic approach to investigate the contribution of CHD to oxidative DNA damage which may affect the functioning of the genes leading to secondary disease manifestations.

Aims and objectives

1. To study the chromosomal aberrations in children with dysmorphism, developmental delay and CHDs.
2. To screen for sequence variants in *GATA4* and *NKX2.5* genes in patients suffering from CHDs.
3. To analyze the magnitude of oxidative DNA damage in the peripheral blood lymphocytes of CHD patients.

CHAPTER 4

General Materials and Methods

The subjects in this study were the patients referred by clinicians of R. L. Jalappa Hospital & Research Centre, the teaching hospital of the Sri Devaraj Urs Medical College, Kolar for chromosomal / molecular genetic analysis either due to frank or suspected genetic diseases. This study was carried out at the Division of Genomics, Department of Cell Biology and Molecular Genetics, Sri Devaraj Urs Academy of Higher Education and Research (SDUAHER), Kolar. Written informed consents were obtained from parents / guardians of the children involved in the genetic studies. Ethics approval for the study was obtained from Institutional Ethical Committee and the study was compiled with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration.

Chemicals and Reagents

All the chemicals and reagents used for the study were of analytical grade obtained from GIBCO, Merck, Qualigens, and Himedia.

1. Metaphase Chromosome preparation

Cytogenetic analyses were performed by standard protocol according to the standard procedure of Hungerford (218). Peripheral blood was collected into sodium heparin vacutainer by venipuncture and stored at 4° C. Culturing was carried out by adding 0.5 ml of blood into a sterile 15 ml falcon tube containing 5 ml working RPMI-1640 medium followed by 0.2 ml of Phytohaemagglutinin (PHA) and incubated at 37° C in 5% CO_2. After 67 ½ hrs, cells were harvested by adding 33 µl of 10 µg/ml Colchicine and incubated at 37° C in 5% CO_2 incubator for 30 minutes. Then tubes were centrifuged for 5 minutes at 2500 rpm and supernatant was discarded. Pellet was resuspended with 8 ml of 0.56% KCl by vortexing and incubated for 30 minutes at 37° C in water bath for hypotonic shock. The tubes were centrifuged at 2500 rpm for 5 minutes and supernatant was discarded. Pellet was resuspended by 2 ml of the freshly prepared Ice-cold

Fixative (3:1 Methanol and Glacial Acetic acid) and mixed well. The contents were centrifuged at 2500 rpm for 5 minutes and the supernatant was discarded. Slides were prepared on clean and pre-chilled slides and placed on a hot plate. Chromosome spreads were analyzed by simple and normal giemsa staining for 4 minutes and cell suspension was stored at 4º C.

G -Banding (GTG)

The standard procedure of Seabright (219) was followed. 2 – 3 days aged slides were treated with trypsin solution for 10-15 seconds. Slides were removed from trypsin solution and immediately rinsed in 0.9% NaCl (normal saline) solution to stop trypsin action. Slides were stained in the giemsa stain for 4 minutes and rinsed thoroughly with distilled water. Slides were dried completely and observed under the microscope. Twenty well spreaded and well-banded metaphases were captured under oil immersion (100x) objective using Carl Zeiss AxioImager A2 (Germany) and 5 were analyzed using the IKAROS (Karyotyping Software). The chromosome abnormalities were identified and designated as per ISCN (2009) nomenclature.

2. Fluorescence *in situ* Hybridization (FISH)

Pretreatment procedure was carried out on fixed cell suspension and slides were prepared using standard cytogenetic procedures. Slides were incubated with protease buffer [Without Pepsin] (10 mM HCl, pH ≈ 3) at 37° C for 30 minutes and it was treated with 2X SSC pH 7.4 at 73° C for 2 minutes. 25 mg of pepsin powder (2500 – 3000 units/mg or 1:60,000) was added to pepsin buffer and mixed thoroughly. Slides were incubated in protease solution for 10 minutes at 37° C and washed in PBS for 5 minutes at room temperature. Then slides were washed in 1% Formaldehyde (Mix together 12.5 ml of 10% neutral buffer formalin, 37 ml of 1X PBS, and 0.5 ml of 100x $MgCl_2$) for 5 minutes at room temperature, followed by 1X PBS wash for 5 minutes at room temperature. Slides were dehydrated for 1 minute in 70% ethanol, 1 minute in 85% ethanol

and 1 minute in 100% ethanol. Probes are prepared by mixing 7 µl of Hybridization Buffer, 1 µL of DNA probe with 2 µl purified H_2O for the total of 10 µl reaction mix. The slides were subjected to co-denaturation and hybridization at 73° C for 5 minutes and 37° C for 16 hours. 10 µl of probe mix was added to the slide and covered with coverslip. After hybridization, slides were washed with wash buffer with 0.4X SSC/0.3% NP-40 and placed into the 73±1° C water bath for at least 30 minutes. Slides were treated with wash buffer 0.4X SSC/0.3% NP-40 for 2 minutes followed by 2X SSC/0.1% NP-40 wash at room temperature for 1 minute. Following this slides were dried in darkness for 10-15 minutes. 10 µl of counterstain (DAPI) was added to the slide and covered with coverslip. Slides were incubated at room temperature for 10 minutes in dark and viewed by using a suitable filter set. Fifty to hundred well spreaded interphases or metaphases were captured under oil immersion (100x) objective using Carl Zeiss AxioImager A2 (Germany) and analyzed using the ISIS Software. The chromosomes were examined and abnormalities noted were identified and designated as per ISCN (2009) nomenclature.

3. DNA extraction from whole peripheral blood (Salting out method)

The standard procedure of standard salting out method was followed (220). Blood samples were collected from patients into sterile EDTA vacutainer and were stored 4° C until processing. Genomic DNA was isolated from the peripheral blood by salting out method. Red blood cells were removed by repeated osmotic shock treatment. To do this, 4 volumes of erythrocyte lysis buffer (ELB) was added to the blood and vortexed. The tubes were kept on ice for 30 minutes to facilitate hemolysis. The samples were then centrifuged at 3000 rpm for 10 minutes and the supernatant was

discarded. The pellet was re-suspended in 2.5 ml of ELB, vortexed and brought up to 10 ml with additional ELB. The sample was centrifuged at 3000 rpm for 10 minutes. ELB treatment was repeated two more times. The white pellet was suspended in 1.8 ml of ELB and vortexed again. To this ELB was added to make up the volume up to 5 ml. 270 µl of 20% SDS and 30 µl of proteinase K (10 mg/ml) were added and mixed .Samples were incubated at 37° C in water bath overnight.

The next day, 500 µl of 5M NaCl followed by equal volume of isopropyl alcohol were added to the lysate. The tubes were swirled to force the resultant silky and mucoid threads of DNA into a globular mass. DNA precipitate was transferred to 0.5 ml of freshly prepared 80% ethanol and incubated at room temperature for 15 minutes. It was centrifuged at 12000 rpm for 5 minutes .the supernatant obtained was discarded, and this step was repeated for three times to obtain purified form of DNA. The DNA was then air dried and dissolved in 500 µl of Tris-EDTA (TE) buffer and then incubated at 65° C for 30 minutes, and kept on rotator until it is dissolved in TE buffer. The dissolved fraction was refrigerated at 4° C for one day and stored at -20° C until use.

DNA quantification

The quality and quantity of the DNA samples were assessed by UV spectrophotometric method by using Perkin Elmer (Lambda 35). 50 µl of TE buffer was pipetted into quartz cuvette and subjected for auto zero correction. 48 µl of TE buffer and 2 µl of DNA sample were added in cuvette, the absorbance was measured at 260 and 280 nm. The absorbance at 260 nm gives DNA concentration and the ratio between 260/280 gives the purity of DNA. DNA samples with 260/280 absorbance ratio between 1.7-1.9 were considered for PCR procedure. DNA samples of expected purity were used for PCR procedure.

4. PCR & DNA Sequencing

The referential genomic DNA sequence of *GATA4* gene was retrieved from Genbank (Accession N0. NC_000008) and *NKX2-5* gene (Accession N0. NC_000005.10). Sequence specific primer pairs were designed to amplify the coding exons and exon-intron boundary regions of *GATA4* and *NKX2-5* gene with the help of Primer Quest tool, IDT DNA software. Polymerase chain reaction (PCR) was carried out with these specific primers and the reactions contained 100ng of genomic DNA, 10X PCR buffer, 10 mM dNTPs, 10 picomole of each primer, 1.5 mM $MgCl_2$, and 1 unit *Taq* DNA polymerase (Bangalore Genei, India) and the conditions followed with an initial denaturation at 95° C for 5 minutes followed by 33 cycles of denaturation at 95° C for 30 seconds, annealing at 61°C for 30 seconds, 72°C for 30 seconds – 1 minute and final extension at 72°C for 10 minutes. The PCR products were purified with GeneJET PCR Purification kit (Thermo Fisher Scientific).

DNA sequencing was performed for all the six exons of *GATA4* and two exons of *NKX2-5* gene with BigDye Terminator v3.1 Cycle Sequencing Kit using ABI-3500 Genetic Analyzer (Applied Biosystems, Foster City, California, USA) according to the manufacturer's instructions. Briefly, sequencing reaction mix along with the template was subjected to amplification followed by cleanup method. This step was followed by the addition of 125 mM EDTA and 3M sodium acetate (pH 4.6) and ethanol mix. After incubation of room temperature, spun down for 5 minutes and add 250 µl of 75% ethanol. Centrifuge it for 10 minutes and decant the supernatant and allow it to dry for 15 minutes. Hi-di Formamide was added to the tubes and subject it to denature and snap chill. Mix the tubes thoroughly and subject it to sequencing. DNA Sequences were analyzed by ABI Variant Reporter software version 1.1 using NG_008177 gene sequence as template for *GATA4* and NG_013340.1 gene sequence for *NKX2-5* gene.

CHAPTER-5

Cytogenetic and Molecular-genetic studies on children with dysmorphism, developmental delay and congenital heart defects

Introduction

Congenital anomalies occur in 2-3% of the live born infants and 20% of still born fetuses and have emerged as an important cause of pediatric mortality and morbidity (62-66,221). Congenital anomalies have not received expected attention in India, particularly in rural population due to the absence of adequate facilities for diagnosis and treatment. Therefore, precise data on the prevalence of congenital anomalies in India is not available as many cases remain unrecorded and efforts in this direction also have not been very well organized. There are good numbers of studies conducted in the western population to unravel the molecular underpinnings of the congenital diseases (71-74,222). In comparison not many studies have been attempted in India. Thus, there is a wide gap between these disease burden and information on their etiology, to assist the diagnostic and management approaches to this otherwise neglected but serious health problem.

The technological revolution has made significant contribution to facilitate the analytical capabilities in molecular genetics to compliment the cytogenetic findings. Array CGH as a tool towards molecular genetic analysis to understand the molecular aberrations in genetic diseases is a rapidly developing trend as evidenced by a large number of reports from several countries (71-76,222). The aim of this study is to systematically probe the cytogenetic and molecular basis of congenital anomalies utilizing combinations of conventional and advanced cytogenetic and molecular biology techniques to unravel the underlying causes in some of the rare and frequently observed congenital anomalies.

Materials and methods

The subjects in this study were the patients referred by clinicians of R. L. Jalappa Hospital & Research Centre, the teaching hospital of the Sri Devaraj Urs Medical College, Kolar for chromosomal / molecular genetic analysis either due to frank or suspected genetic diseases. This study was carried out at the Division of Genomics, Department of Cell Biology and Molecular Genetics, Sri Devaraj Urs Academy of Higher Education and Research (SDUAHER), Kolar. Written informed consents were obtained from parents / guardians of the children involved in the genetic studies.

A total of 431 subjects clinically diagnosed or suspected for genetic abnormalities / congenital anomalies were included in this study. The system wise anomalies observed and provisional diagnoses as well as the number of cases are detailed in Table 5-1.

Inclusion Criteria

- Children with multiple congenital anomalies, dysmorphism, developmental delay and CHD
- Other chromosomal and genetic syndromes

Exclusion Criteria

- Children without any congenital anomalies, dysmorphism, developmental delay and CHD

Cytogenetic and molecular analysis

Cytogenetic analyses of the samples were performed by standard protocol (218) in order to identify the numerical and structural aberrations. Fluorescent *in situ* Hybridization (FISH) was also carried out to confirm the numerical and chromosomal aberrations. Genomic DNA was extracted from blood sample using salting out method (220). DNA concentration and purity was checked by optical density ratios (260/280 nm) using UV-Spectrophotometer. The samples were stored at - 20°C until use. Array CGH was performed according to manufacturer protocols.

Results

A total of 431 cases / subjects were included in this study. Samples from all the subjects were subjected to karyotyping. A comparative analysis of the system wise congenital anomalies showed two abnormal karyotype with urogenital abnormalities namely Pseudo-hermophroditism and Ambiguous genitalia. In addition to these two cases, three cases of global developmental delay also showed chromosomal aberrations. The data on system wise congenital anomalies and cytogenetic investigations is detailed in Table 5-1.

Table 5-1: System wise congenital anomalies investigated in various systems.

System	Congenital anomalies	No. of cases	No. of cases with chromosomal aberrations	Percentage of cases
Craniospinal	Anencephaly	2	-	**62 (14.39%)**
	Meningomyelocele	3	-	
	Spina bifida	1	-	
	Hydrocephalus	8	-	
	Microcephaly	31	-	
	Brachycephaly	1	-	
	Megalencephaly	1	-	
	Macrocephaly	2	-	
	Acrania	1	-	
	Kyphoscoliosis	3	-	
	Craniosynostosis	5	-	
	Hypochondroplasia	1	-	
	Dandy walker syndrome	3	-	
Eye & Ear	Microphthalmos	2	-	**4 (0.93%)**
	Microcornea	1	-	
	Bilateral microtia	1	-	
Cardiovascular	Atrial septal defect (ASD)	28	-	**69 (16%)**
	Ventricular septal defect (VSD)	12	-	
	Patent ductus arteriosus (PDA)	6	-	
	Pulmonary valve stenosis (PS)	2	-	
	Pulmonary valve atresia	1	-	
	Tetralogy of Fallot (TOF)	1	-	
	Double outlet right ventricle (DORV)	1	-	
	ASD, VSD	1	-	
	ASD, PDA	4	-	
	ASD, VSD, PDA	3	-	
	Complex CHD	10	-	
Musculoskeletal	Clinodactyly	7	-	
	Polydactyly	2	-	
	Syndactyly	4	-	

	Brachydactyly	2	-	**30 (6.96%)**
	Hypertrophy of right upper limb	1	-	
	Dysmorphology in upper, lower limb	1	-	
	Hip dislocation	1	-	
	Achondroplasia	1	-	
	Amniotic band sequence	1	-	
	Cleft lip	2	-	
	Cleft palate	4	-	
	Cleft lip with cleft palate	4	-	
Bone	Osteoporosis	1	-	**3 (0.69%)**
	Osteogenesis imperfecta	2	-	
Respriatory	Aplasia of lung	1	-	**1 (0.23%)**
Gastro-intestinal	Oesophageal atresia	1	-	**5 (1.16%)**
	Duodenal atresia or stenosis	2	-	
	Gastroschisis	1	-	
	Omphalocele	1	-	
Urogenital	Polycystic kidney*	3	-	**33 (7.66%)**
	Renal agenesis	2	-	
	Undescended testicle	7	-	
	Hypogonadism	1	-	
	Pseudo-hermophroditism	1	1	
	Micropenis	12	-	
	Hypospadias	2	-	
	Ambiguous genitalia	3	1	
	Congenital hydronephrosis	1	-	
	Absent vaginal orifice	1	-	
Others	Global developmental delay	29	3	**36 (8.35%)**
	Failure to thrive	7	-	

* Adult samples included as part of screening studies

Similarly, comparative analysis of provisional clinical diagnosis and cytogenetic studies of chromosomal syndromes, and other syndromes and various other clinical conditions helped to rule out the ambiguities primarily in the cases of Down, Turner and Edwards syndromes. Out of the 51 clinically suspected Down syndromes, only 24 of them were turned out to be fitting in to

classical Down syndrome. In the case of Turner syndrome, out of 7 cases, 4 were cytogenetically proved true Turner and for Edwards syndrome, 1 case out of 2 proved positive as detailed in Table 5-2. Table 5-3 provides details of various types of chromosomal abnormalities pictured in this study.

Table 5-2: Chromosomal syndromes, other syndromes and various clinical conditions carried out for cytogenetic analysis.

	Clinical conditions	No. of cases	No. of cases with chromosomal aberrations	Percentage of cases
Chromosomal Syndromes	Down syndrome	51	24	**67 (15.55%)**
	Turner syndrome	7	4	
	Edwards syndrome	2	1	
	Pataus syndrome	1	-	
	Prader willi syndrome	3	-	
	Cri-du chat syndrome	1	-	
	Klinefelter syndrome	1	-	
	1p36 Microdeletion syndrome	1	1	
Other syndromes & clinical conditions	Crouzan syndrome	2	-	**71(16.47%)**
	Treacher collins syndrome	2	-	
	Cerebral palsy	3	-	
	Colloidon baby syndrome	3	-	
	Noonan syndrome	4	-	
	Marfans syndrome	2	-	
	Fanconi anemia	4	2	
	Stickler syndrome	1	-	
	Hunter syndrome	1	-	
	Cystic fibrosis*	1	-	
	Multiple exostosis	6	-	
	Pierre robin syndrome	1	-	
	Cornelia de Lange syndrome	1	-	
	Dwarfism*	1	-	
	Progeria	1	-	
	Bartters syndrome	1	-	

	Spinal muscular atrophy	3	-	
	Thalassemia major	3	-	
	Leukemia (CML / AML)*	6	-	
	Cerebral ataxia*	2	-	
	Ehlers-Danlos syndrome	1	-	
	Cystic hygroma	1	-	
	Marshall syndrome	1	-	
	Attic syndrome	1	-	
	Moebius syndrome	1	-	
	Congenital hypothyroidism	3	-	
	Retinitis pigmentosa	2	-	
	Myelodysplastic syndrome*	2	-	
	Leighs disease	1	-	
	Puetz Jeghers syndrome	1	-	
	Febrile thrombocytopenia	1	-	
	Facio-auriculo vertebral spectrum	1	-	
	Hemangiopericytoma	1	-	
	Metachromatic leuko dystrophy*	1	-	
	Dyschromatosis congenita	1	-	
	Duchenne muscular dystrophy	2	-	
	Arthrogryposis multiplex congenita	1	-	
	Brugada syndrome	1	-	
	Parental karyotyping	50	1	**50 (11.6%)**

* Adult samples included as part of screening studies

Table 5-3: Chromosomal abnormalities picked up from 431 cases subjected to karyotyping.

S.No.	Clinical conditions	No. of cases with chromosomal abnormalities
1.	Down syndrome	24
2.	Turner syndrome	4
3.	Partial Trisomy 18	1
4.	48,XXYY syndrome	1
5.	Partial trisomy 14q & 12p	1
6.	1p36 microdeletion syndrome	1
7.	46,XY (abnormal karyotype)	2

8.	Others	4
TOTAL		**38**

Numerical changes observed in the karyograms in the cases of Down, Turner, Edwards and 48,XXYY syndromes are shown in Figure 5-1.

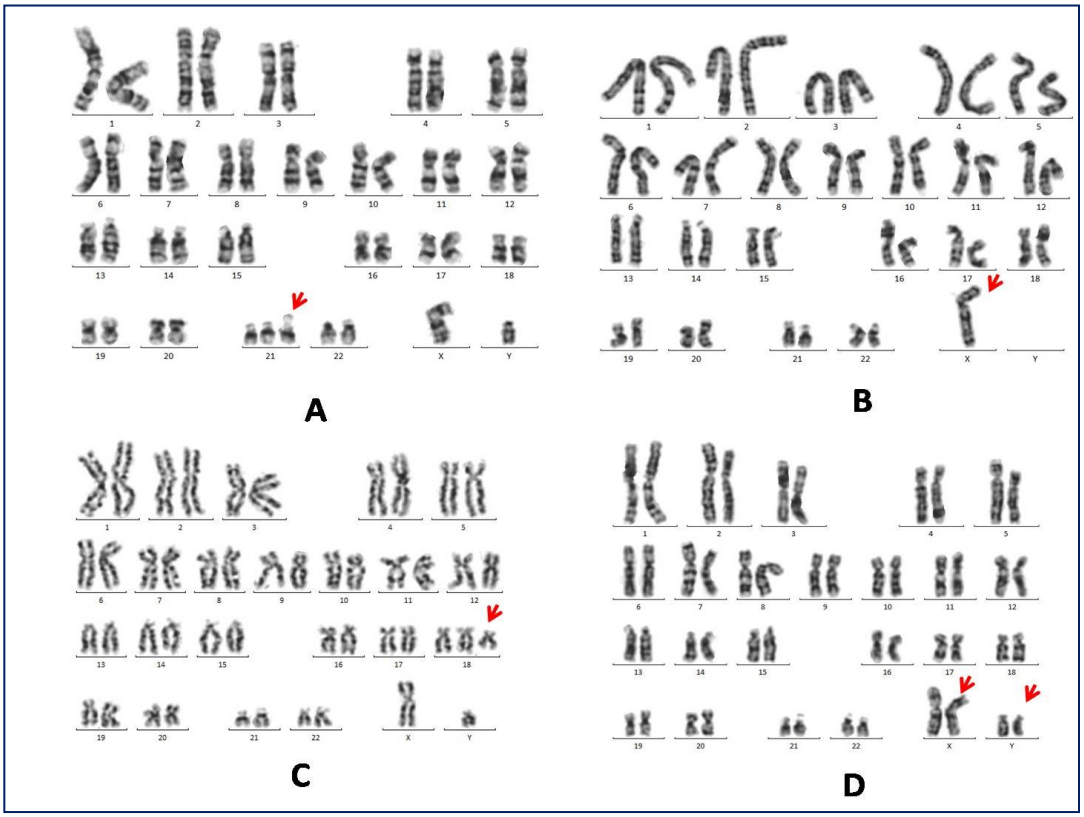


Figure 5-1. Karyotypes of (A) 47,XY,+21 (Down syndrome); (B) 45,X (Turner syndrome); (C) 47,XY,+18 (Partial trisomy 18); (D) 48,XXYY syndrome.

Apart from numerical chromosomal changes, cytogenetic studies also resulted in picking up of chromosomal aberrations in the following cases of Partial trisomy 14q and 12p, balanced translocation of 14q to 12p, balanced translocation of 21q to 14p and rare balanced translocation of 13q to 12p as shown in the Figure 5-2.

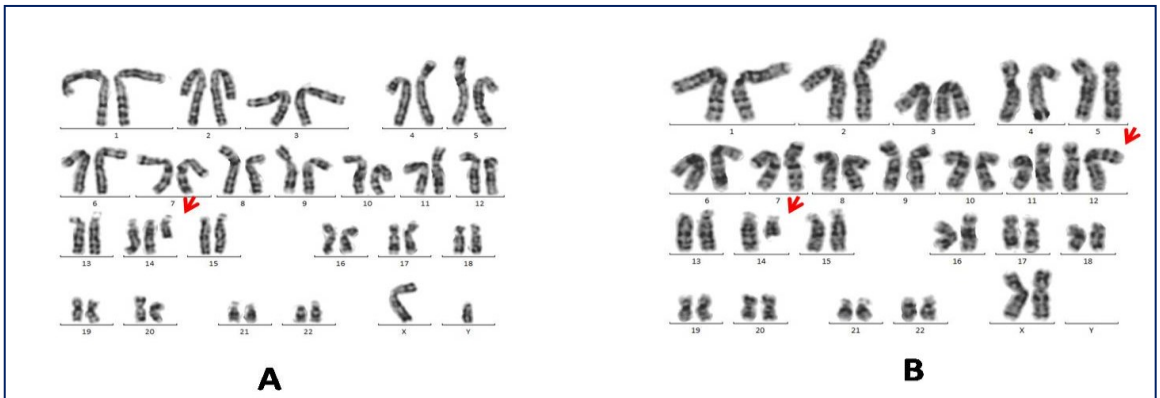


Figure 5-2. Karyotypes of (A) Partial trisomy 14q and 12p; (B) Balanced translocation from 14q to 12p; (C) Balanced Robertsonian translocation from 21q to 14p; (D) Rare translocation from 13q to 12p.

In order to confirm the numerical chromosomal changes and chromosomal aberrations, Fluorescent *in situ* Hybridization studies were conducted in few cases and confirmed the cases of Down, Turner and one case of 1p36 micro-deletion and a case of 48,XXYY syndrome as shown in Figure 5-3.

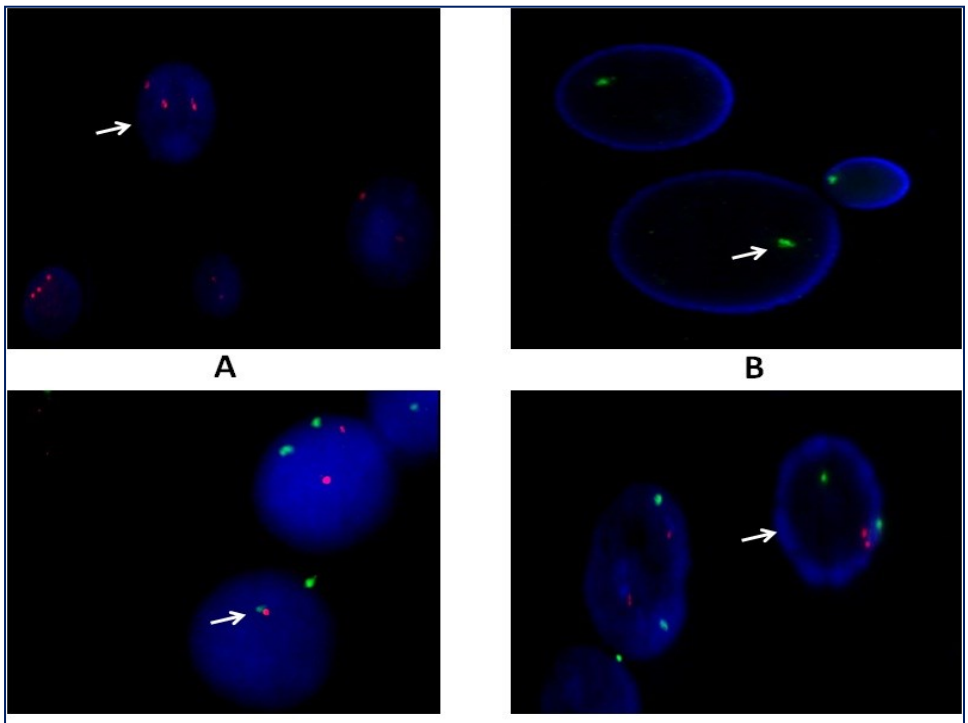


Figure 5-3. FISH images A - Down Syndrome confirmed with locus specific probe, Kreatech, DSCR1 covering markers D21S65, RH72110 and RH92717 (red signal); B-Turner Syndrome confirmed with X and Y Enumeration probe, Cytocell, DXZ1 (green signal) of X centromeric region; C –1p36 microdeletion syndrome with Monosomy 1p probe, Cytocell, SKI probe covering SKI gene and MORN1 gene with marker D1S2515 (green signal) for 1p36 region and control probe for 1qter region (red signal) & D - 48,XXYY syndrome confirmed with X and Y Enumeration probe, Cytocell, DXZ1 (green signal) and DYZ3 (red signal) of X and Y centromeric region.

Close examination of the karyograms of Turner syndrome yielded one interesting case with the involvement of both numerical and structural abnormalities in the X chromosome. The numerical changes in the chromosome were observed by GTG banding and C-banding. These observations were further confirmed by subjecting it for FISH analysis. The results of these studies depicted in the Figure 5-4.

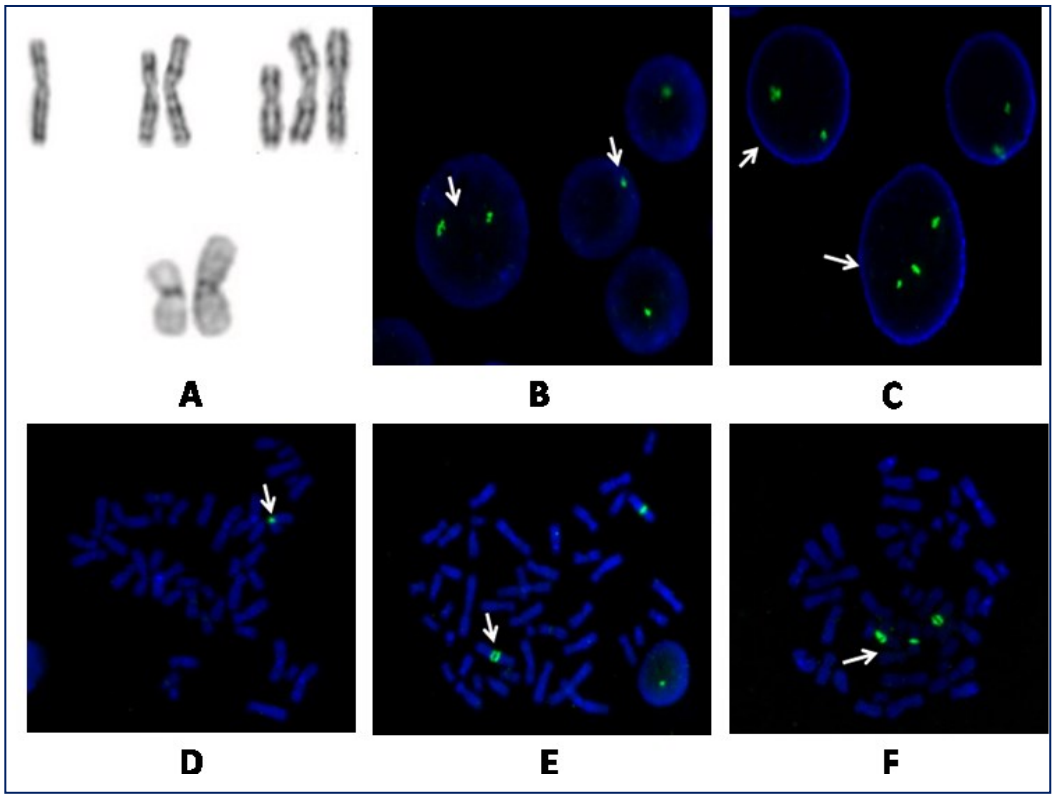


Figure 5-4. A-Proband's karyogram (GTG banding) showing three cell lines (upper lane) of Turner syndrome 45,X/46,X,idic(X)(p11.2)/47,X,idic(X)(p11.2),+idic(X)(p11.2) and C-banding (down lane) of isodicentric X chromosomes; B and C - Interphase FISH image of 3 different cell lines; D – F Metaphase FISH image of 3 different cell lines.

The study group had good number of cases with dysmorphic features with normal karyotype. Three of such cases were subjected to array comparative genomic studies to assess the genotype-phenotype correlations.

CASE 1:

A 5 year old male child with dysmorphic features like microcephaly, low set ears with prominent pinnae, and nose with a broad nasal bridge, high arched palate besides retrognathia with mental retardation. He was diagnosed for Atrial Septal defect (ASD) and pulmonary stenosis along with global developmental delay. The proband's karyotype was 47,XY,+der(14)t(12;14)(p13;q22)mat and parental chromosomal analyses revealed that the mother was a carrier of a balanced translocation 46,XX,t(12;14)(p13;q22) and the father was normal 46,XY. Array CGH analysis of proband confirmed the partial trisomy 14q with the amplification of 32313772 bases in q11.1 to 22.1 region of Chromosome 14. Chromosome 12 also showed amplification of 1000846 bases in the terminal region (p13.3 – pter) indicative of partial trisomy of 12p. Chromosomal analysis of the parents also confirmed the maternal origin of marker chromosome (14q-) in the proband. The proband's karyotype was 47,XY,+der(14)t(12;14)(p13;q22)mat and the mother's karyotype was 46,XX,t(12;14)(p13;q22) (Figure 5-5 A & B).

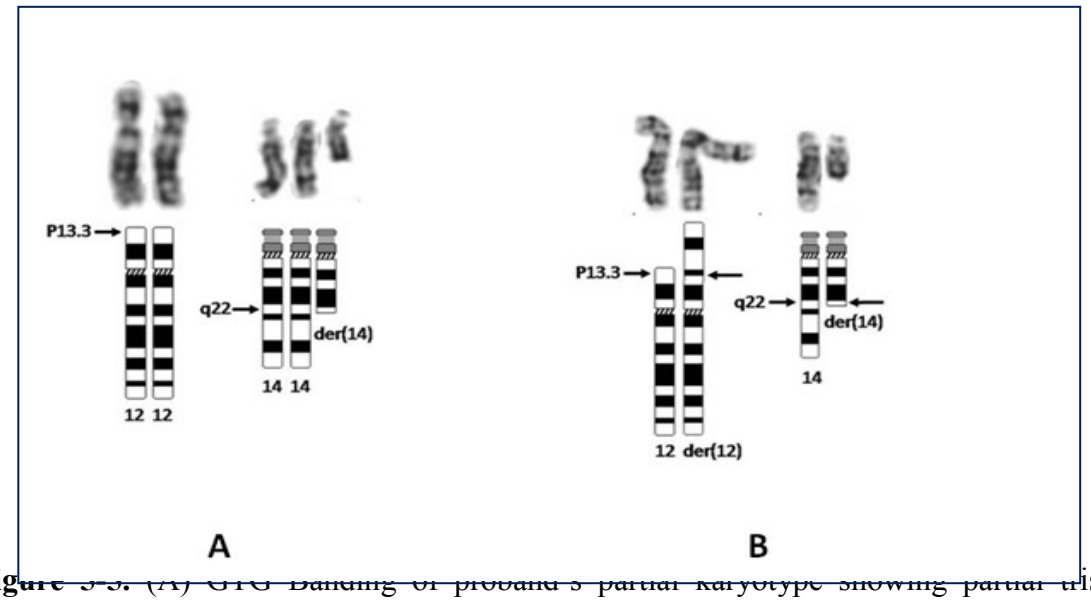


Figure 5-5. (A) GTG Banding of proband's partial karyotype showing partial trisomy 14q 46,XY,+der(14)t(12;14)(p13;q22)mat. Arrows pointing to the chromosomes 12 and 14 are translocation breakpoints. (B) Mother's partial karyotype showing balanced translocation 46,XX,t(12;14)(p13;q22). Arrows pointing to the chromosomes 12 and 14 are translocation breakpoints and break-rejoin junction.

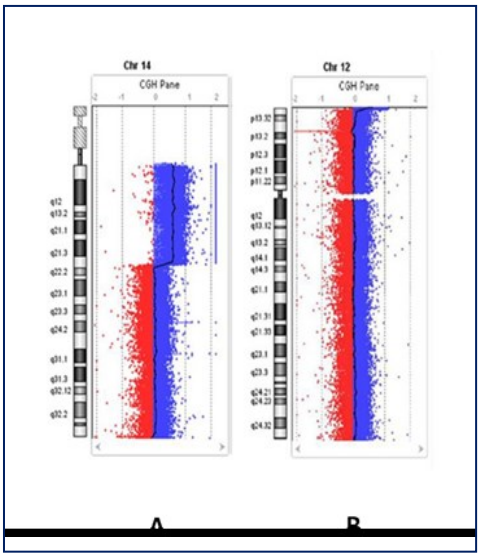


Figure 5-6. (A) Array CGH profile revealing partial trisomy 14q with amplification in 14q11.1-q22.1 region. (B) partial trisomy 12p with amplification in 12p13.3-pter region.

In proband, array CGH analysis confirmed the partial trisomy 14q with the amplification of 32313772 bases (18505611 – 50819383) in q11.1 to 22.1 region of Chromosome 14 represented by 10312 probes on the arrays. Chromosome 12 also showed amplification of 1000846 bases

(59822 – 1060668) in the terminal region (p13.3 – pter) represented by 436 probes suggesting partial trisomy of 12p (Figure 5-6 A & B). This partial trisomy 12p segment was undetectable by conventional karyotyping as the amplified segment was about 1 Mb.

CASE 2:

The subject was a 8 days old female child presented with week cry, microcephaly, short neck, bilateral microtia and other dysmorphic features. She was the second child born to a consanguineous couple. Her birth weight was 2.75 kg at term delivery. Physical examination revealed that the head circumference 32.5 cm and weight 2.32 kg. Clinical examination revealed dysmorphic features like round face with low set bilateral microtia, mild left hearing loss, microcephaly, mongoloid slant, hypertelorism and retrognathia. Chromosomal analysis showed a normal karyotype (46,XX) for the child. The parental karyotyping also provided normal picture.

Follow up at the age of 1 year 10 months; child was brought with the complaints of global developmental delay. Her weight was 6.98 kg (3^{rd} centile), head circumference 48 cm (3^{rd} centile) and height was 75 cm (3^{rd} centile). CT brain examination was normal. Clinical examination showed up alon with epicanthic fold, hypotonia, squint, single simian crease, small toe, broad nasal tip, hyper extensible joint and slight left hearing loss. At the age of 2 years 6 months, her head circumference was 40.25 cm and height was 78 cm with the above said complications. Other systems like chest, spine and genitalia were normal. No other significant family history.

Array CGH was performed using an Affymetrix CytoScan™ 750K array. Microarray analysis showed a homozygous deletion involving chromosome 8 (832 Kb deleted) within 8q22.2, indicating nullisomy for this region (Figure 5-7). This region covering from 100,021,531 to 100,853,542 bases in which most of the exons (exon 1 – 54) of *VPS13B* gene were deleted. Features of the patient were overlapping with other ethnic patient phenotypes along with

retrognathia, overidding suture, bilateral microtia and hearing loss. The absence of heterozygosity increased the suspicion for a recessive genetic condition mapping to these intervals and it is recommended for clinical correlation of these findings.

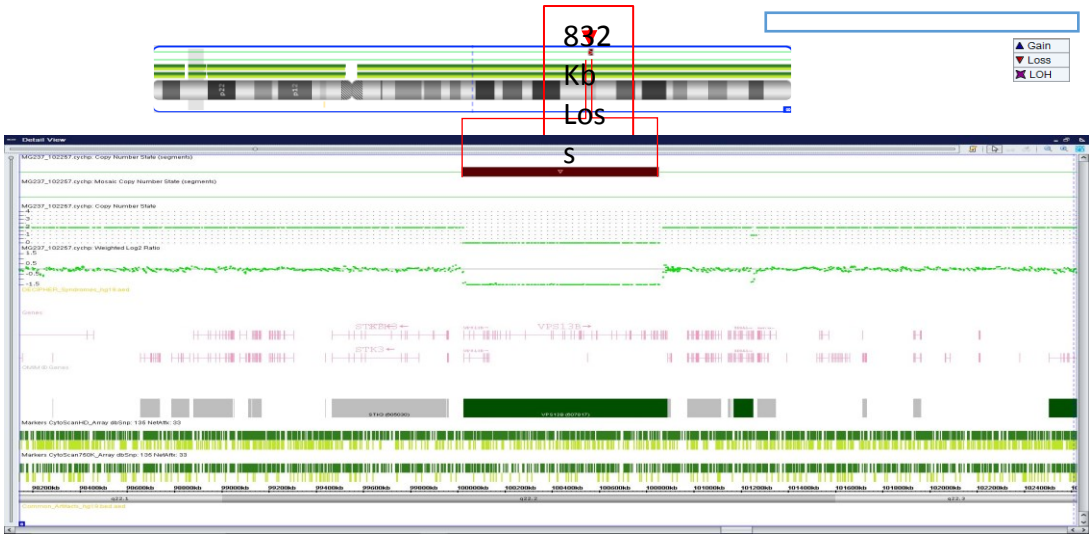


Figure 5-7. Array-CGH showing homozygous deletion in *VPS13B* gene of chromosome 8q (832 Kb).

CASE 3:

A 9 month old female child was presented with microcephaly, hypotonia, rocker bottom feet, clinodactyly and dysmorphic features like low set ears, long philtrum, and triangular face with small mouth, nose and thin upper lip along with simian crease. The karyogram of this subject presented the normal picture of 46,XX, thus warranting the need for better analytical techniques

to understand the molecular defect. Therefore microarray analysis was performed and the results did not yield any chromosomal aberrations.

Discussion

Genetic diseases as well as congenital anomalies are challenges to health care professionals as these are clinical problems which could not be addressed as the diagnosis, treatment / management of these diseases require a plethora of investigations primarily at molecular levels. The advent of gene therapy has been found useful in some of the genetic disorders, but it is yet to be utilized regularly for reasons such as non-affordability and the clinical settings and other facilities required. The inadequacies in the diagnosis and treatment of genetic / congenital anomalies however have not dampened the search for the underlying causes of the diseases as evidenced for the large number of investigators engaged in research in this area. Further, outcome of these studies could aid to the management of the genetic problem and its perpetuation through genetic counseling and awareness programs. As a preliminary step of genetic analysis of the various types of genetic diseases presented in the study subjects, all of them underwent cytogenetic analysis irrespective of the known causes of the genetic disease manifestation. Further, this study focused on the molecular basis of some of the congenital anomalies seen among the patients of this region through a systematic approach blending the conventional karyotyping /cytogenetic evaluation with the advanced technologies like array CGH.

This study considered 431 cases either frank or suspected for genetic diseases / congenital anomalies referred by clinicians for cytogenetic or molecular analysis to correlate the phenotypic features with the genotype of the subjects. Anomalies of all the organ systems were screened and majority was of cardiovascular anomaly to the extent of 16%, followed by craniofacial 14.39%, urogenital 7.66%, musculoskeletal 6.96%, gastrointestinal 1.16%, eye and ear 0.93%, respiratory

0.23% and other severe dysmorphic, global developmental delay and failure to thrive cases 8.35% as elaborated in the Table 5-1. Chromosomal syndromes were to the extent of 15.55% and there were several other syndromes and clinical conditions accounting for 16.47%. Parental samples analyzed were to the extent of 11.6% elaborated in the Table 5-2. The subjects involved in this study have some characteristics with respect to their culture, occupation and environment in which they live. Consanguinity, exposure to occupational hazards, adverse environmental factors, socioeconomic, nutritional statuses etc. are very pertinent and of relevance for this unusually large number of congenital anomalies.

The chromosomal analysis of the congenital anomalies involving various systems presented chromosomal aberrations only in three cases with global developmental delay. This include one case of 48,XXYY syndrome similar observation had been reported in the studies of Nicole Tartaglia *et al.,* 2008 and Prasad Katulanda *et al.,* 2012 (223,224). In a study conducted by Yeo-Hyang Kim *et al.,* 2013 reported a case of trisomy 14q (225) and Shackelford AL *et al.,* 2013 had reported trisomy 12p in probands suffering from global developmental delay (226). Partial trisomy 14q and trisomy 12p observed in this study has been unique and first of its kind report in a proband suffering from global developmental delay. In third case we have observed 13q to 12q balanced translocation in comparison to the translocations from 13q to 8p and also from 13q to 3p reported respectively by Rodovalho-Doriqui MJ *et al.,* 2013 and Chih-Peng Chen *et al.,* 2011. It could be seen above data that very few of the global developmental delay attributed to chromosomal aberrations (227,228). However, a large number to the extent of 90% of the global developmental delay did not provide any visible abnormalities by conventional cytogenetics.

This observation has evinced interest to explore the underlying causes for the developmental delay in these cases. Traditional methods of chromosome analysis like karyotyping

and FISH can identify abnormalities upto a limit of ~5MB while FISH is typically limited to few loci and it remains targeted at the detection of suspected imbalance based on clinical suspicion. Consequentially, submicroscopic deletions and amplifications remain unidentified. Array CGH is a first level new technology to the cytogenetic laboratory for the evaluation of genetic disorders and the high resolution data will contribute toward better understanding of genotype-phenotype correlations. It is a high throughput method applied to detect copy number variations to a resolution of even as low as 50 Kb. Thus it was felt appropriate to utilize array CGH technology to explore the possible chromosomal submicroscopic aberrations in global developmental delay.

Study conducted in a proband as detailed in the case 2 under result section yielded a very interesting data wherein array studies picked up a deletion of 832 kb on chromosome 8 which accommodated *VPS13B* gene. This finding is a rare deletion where the underlie cause is majorly point mutations on *VPS13B* gene detected by PCR and MLPA techniques. This gene has been implicated in a rare autosomal recessive disorder Cohen syndrome (CS; OMIM# 216550) characterized by microcephaly, retinal dystrophy, neutropenia, joint hypermobility, truncal abesity, distinctive facial features, intellectual disability and developmental delay. It is the largest gene encompasses of 62 exons located on the chromosomal location of 8q22.2 (229). *VPS13B* gene is also known as *COH1* gene and it is expressed mainly in the central nervous system, blood, muscles and heart.

In the third case of global developmental delay array CGH studies did not provide any deletion or duplication in the chromosomes. The case was characterized by microcephaly, hypotonia, rocker bottom feet, clinodactyly and dysmorphic features like low set ears, long philtrum, and triangular face with small mouth, nose and thin upper lip along with simian crease. It is interesting thus to not that in this case the array CGH did not pick up the underlying cause

warranting further molecular study viz, NGS preferably, whole genome sequencing process to identify the molecular aberrations.

An indepth analysis was carried out in the case 1 of partial trisomy 14q to know the extent of chromosomal aberrations and its pattern. The proband presented with partial trisomy 14q suggesting the need for parental karyotyping. The results indicated balanced translocation of 14q to 12p region in the mother with karyogram of the father presenting normal chromosomes. In order to know the break points and the extent of chromosomal aberrations, array CGH studies were conducted which confirmed partial trisomy 14q and partial trisomy 12p.

Microarray studies helped to establish the genotype-phenotype correlations in this case by extensive search in OMIM database for the amplified genes in those regions (230). This led for considering *MYH6* (OMIM #614089), *FOXG1* (OMIM #164874), *SUPT16H* (OMIM #613457) and *MGAT2* (OMIM #602616) genes which are closely associated with the phenotypic presentations like dysmorphic features, mental retardation and developmental delay (230).

It is evident from the studies detailed that applications of chromosomal analysis either by conventional means (karyotyping) or array CGH beneficial to explain some of the underlying causes of genetic abnormalities. However, it has its limitations in a large number of cases which exhibited system wise anomalies presenting with normal karyograms and without deletion or duplications. Thus, the necessity of better analytical tools was apparently highlighted through this study as has been observed by many other investigators (231,232).

Further, the anomalies considered here were heterogeneous by presenting a plethora of clinical conditions though they were system wise. And each of these conditions could be having specific underlying causes. Though this screening study had very interesting cases presenting

unusual phenotypes there had been no consistency in their forms and in clinical observations could not be confirmed with targeted and specific investigations. Thus, each of these cases had to be considered as isolated situations as attempted and described earlier in this section with some interesting findings. Therefore, it was felt appropriate to confine specific clinical conditions which could be supported by reliable investigations in order to have a meaningful outcome from the current study. Keeping this important aspect in mind further studies were planned and confined to congenital heart diseases. It may be noted that CHD formed relatively a larger group (16%) consolidated in Table 5-1 among the system wise anomalies.

Conclusion

Data presented in this chapter discusses the spectrum of congenital anomalies observed in this region which seemed to be unusually frequent, probably could be attributed to various factors such as cultural, environmental, occupational and nutritional status. This observation proposed for creating of birth registry of dysmorphia with demographic details which would enable to correlate the findings with the probable factors responsible for the congenital anomalies. Though conventional cytogenetic studies were able to pick up a few abnormal chromosomal patterns in cases presented with global developmental delay, it failed to provide cues in majority of cases. Array CGH studies conducted lead to providing information on deletions or duplications and further critical analysis of these regions indicated the candidate genes that were missing or duplicated. This approach has led to the identification of Partial Trisomy 12p and is a first time report. In addition, array CGH failed to show up any aberration in a case led to the conclusion that molecular level studies necessary in most of the patients of congenital anomalies to identify the cause of the disease condition.

CHAPTER-6

Mutational analysis of *GATA4* and *NKX2-5* gene in patients suffering from Congenital Heart Diseases

Introduction

Development of human heart is a complex, multifactorial event controlled by multiple genes and biological process. Defect in these processes leads to the formation of various Congenital Heart Disease (CHD) conditions and is one of the most common birth defects in newborns that arise before birth. CHD is a complex multifactorial disorder with genetic and environmental factors playing an important role in disease development (10). Genetic and environmental factors (80%) play multifactorial role in the etiology of CHD with remaining 20% attributed to mendelian syndromes, single gene disorders and teratogens (233). Reports support that mutation in transcription factors *GATA4*, *NKX2-5*, *TBX5*, *MYH6* and others are primarily responsible for the development of CHD (21-23). *GATA4* and *NKX2-5* transcription factors play an important role in foetal heart development. Sporadic and germline mutations in these genes have been shown to be associated with various CHD conditions (24,84). However, the genetic basis of CHDs has not been explored in depth in the Indian population. It was worthwhile to consider and investigate the sequence variants in *GATA4* and *NKX2-5* genes in a wide spectrum of various CHDs.

Materials and methods

Subjects

The subjects included in this study were the patients referred by clinicians of R. L. Jalappa Hospital & Research Centre and Narayana Hrudayalaya heart centre, Kolar for molecular analysis. A total of sixty patients with a spectrum of CHDs were included in the study (Table 6-1). Pediatric cardiologists confirmed CHDs by two-dimensional echocardiography using color flow doppler. Whole peripheral blood samples were obtained from the CHD patients for molecular analysis of *GATA4* and *NKX2-5* gene. The Institutional Ethics Committee has approved the study and written informed consents have been obtained from all the patients.

DNA extraction

Genomic DNA was extracted from 2 ml of peripheral blood sample collected into sterile EDTA vacutainer using standard salting out method (220). DNA concentration and purity was checked by optical density ratios (260/280 nm) using UV-Vis Spectrophotometer (Lambda 35, Perkin Elmer, Waltham, Massachusetts, USA) and stored at -20°C until use.

Table 6-1. Baseline characteristics of study subjects *(n=60).*

Characteristics	Gender		Total
	Male	**Female**	
Age in Years			
0-5	22 (75.8)	24 (77.4)	46 (76.6)
5-10	2 (6.8)	4 (12.9)	6 (10)
10-15	3 (10.3)	2 (6.4)	5 (8.3)
15-18	2 (6.8)	1 (3.2)	3 (5)
	29	**31**	**60**
Congenital Heart Diseases			
Atrial Septal Defect (ASD)			
-Ostium Secundum ASD	9 (31)	15 (48.3)	24 (40)
Ventricular Septal Defect (VSD)			
-Perimembranous VSD	3 (10.3)	8 (25.8)	11 (18.3)
-Muscular VSD	1 (3.4)	0	1 (1.6)
Patent Ductus Arteriosus (PDA)			
Tetralogy of Fallot (TOF)	2 (6.8)	1 (3.2)	3 (5)
Pulmonary Stenosis (PS)	1 (3.4)	1 (3.2)	2 (3.3)
-Valvar PS	1 (3.4)	0	1 (1.6)
Double Outlet Right Ventricle (DORV)	1 (3.4)	0	1 (1.6)
ASD, VSD	3 (10.3)	1 (3.2)	4 (6.6)
ASD, PDA	7 (24.1)	2 (6.4)	9 (15)
ASD, VSD, PDA	1 (3.4)	3 (9.6)	4 (6.6)
Total	**29(100)**	**31(100)**	**60(100)**

Sequence analysis

The referential genomic DNA sequence of *GATA4* gene was retrieved from Genbank (Accession N0. NC_000008) and *NKX2-5* gene (Accession N0. NC_000005.10). Sequence specific primer pairs (Table 6-2) were designed to amplify the coding exons and exon-intron boundary regions of *GATA4* and *NKX2-5* gene with the help of Primer Quest tool, IDT DNA software. Polymerase chain reaction (PCR) was carried out with these specific primers and the reactions contained 100ng of genomic DNA, 10X PCR buffer, 10 mM dNTPs, 10 picomole of each primer, 1.5 mM $MgCl_2$, and 1 unit *Taq* DNA polymerase (Bangalore Genei, India) and the conditions followed with an initial denaturation at 95°C for 5 min followed by 33 cycles of denaturation at 95°C for 30 sec, annealing at 61°C for 30 sec, 72°C for 30 sec – 1 min and final extension at 72°C for 10 min shown in Figure 6-1. The PCR products were purified with GeneJET PCR Purification kit (Thermo Fisher Scientific). Sequence analysis was performed for all the six exons of *GATA4* and two exons of *NKX2-5* gene with BigDye Terminator v3.1 Cycle Sequencing Kit under ABI-3500 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). DNA Sequences were analyzed by ABI Variant Reporter software version 1.1 using NG_008177 gene sequence as template for *GATA4* and NG_013340.1 gene sequence for *NKX2-5* gene.

Table 6-2 List of *GATA4* and *NKX2-5* gene primers

***GATA4* primers**			
Exon	**Forward primer (5' to 3')**	**Reverse Primer (5' to 3')**	**Annealing temperature**
Exon 2	GAACTCTCAGTGTCTGGGATTAG	GTGGCTCCAGCTAACTCTAAA	61°C
Exon 3	TGACGGTGAATGATGGTTAGG	GGCCAGCAAAGTAGTTGAAAG	61°C
Exon 4	CATCACACAGGTGCTCGATAAG	CCAAAGATGAAAGGACCGAGTA	61°C
Exon 5	TGTAGCCCTCCGCAGATAA	GTCATGTCCACATCACCATCT	61°C
Exon 6	TTCTGGGCAACCACAGTATC	AGTCCCATCAGCGTGTAAAG	61°C
Exon 7	GCTCCTTCACTTCCAACATCTC	ACCCTCTCCCAGGAAATTAAAG	61°C
***NKX2-5* primers**			
Exon 1	GCTCATCGCTCCTGTCATC	CGACAACACCAGGCATCTTA	61°C
Exon 2-1	CACGAGGATCCCTTACCATTAC	CTCATTGCACGCTGCATAATC	61°C
Exon 2-2	AGTTTGTGGCGGCGATTA	GCTCGCAGGTAAGTCATTAAAC	61°C

Multiple sequence alignments

Protein sequences of GATA4 protein from various species were obtained from NCBI protein database and aligned using MUSCLE program (www.ebi.ac.uk/Tools/msa/muscle/).

Mutation prediction

PolyPhen-2 (Polymorphism Phenotyping, http://genetics.bwh.harvard.edu/pph2/), SIFT (Sorting Intolerant From Tolerant, http://sift.jcvi.org/) and MutationTaster (www.mutationtaster.org) (234) programs were utilized to validate the mutation prediction of *GATA4* gene sequence variants. SIFT and MutationTaster programs were performed to validate the mutation prediction of *NKX2-5* gene sequence variants.

***In silico* analysis of *GATA4* gene**

The intronic mutations, RNA splice sites and RNA branch points of *GATA4* gene were analyzed by using Human Splicing Finder (HSF) (235) software version 3.0 (www.umd.be/HSF3/), an online bioinformatics tool to predict the effects of mutations on splicing signals or splicing motifs either to enhance or suppress splicing such as Exonic Splicing Enhancers (ESEs), Exonic Splicing Silencers (ESSs), Intronic Splicing Enhancers (ISEs) and Intronic Splicing Silencers (ISSs).

Results

***GATA4* gene analysis**

The patient population with CHD included in this study was 60 and 10 samples without CHD were included as controls in the study. A total of 60 patients consisting of 24 ostium secundum ASD, 11 perimembranous and 1 muscular VSD, 9 ASD with Patent Ductus Arteriosus (PDA), 4 ASD and VSD, 4 ASD, VSD and PDA, 3 PDA, 2 TOF, one each with Valvar Pulmonary Stenosis (PS) and Double Outlet Right Ventricle (DORV) in this study as detailed in Table 6-1.

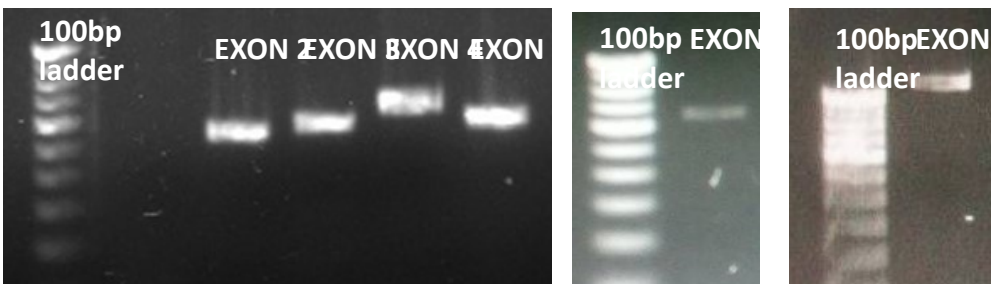


Figure 6-1. Agarose gel electrophoresis image showing PCR amplicons of *GATA4* and *NKX2-5* gene.

Mutational analysis of CHD cases revealed twenty variants including five in the coding (Table 6-3) and fifteen in the non-coding region of *GATA4* gene (Table 6-4 A & B). The data analysis of controls also presented intronic variants however the genotypic pattern were different from the cases under study group (Table 6-5 A & B). There were five known heterozygous missense mutations (p.T355S, p.S377G, p.V380M, p.P394T and p.D425N) identified in exon 5 and exon 6 regions (Figure 6-3 A). Multiple sequence alignment of *GATA4* sequence from various species was aligned and only p.D425N showed the evolutionarily conserved throughout the species (Figure 6-2).

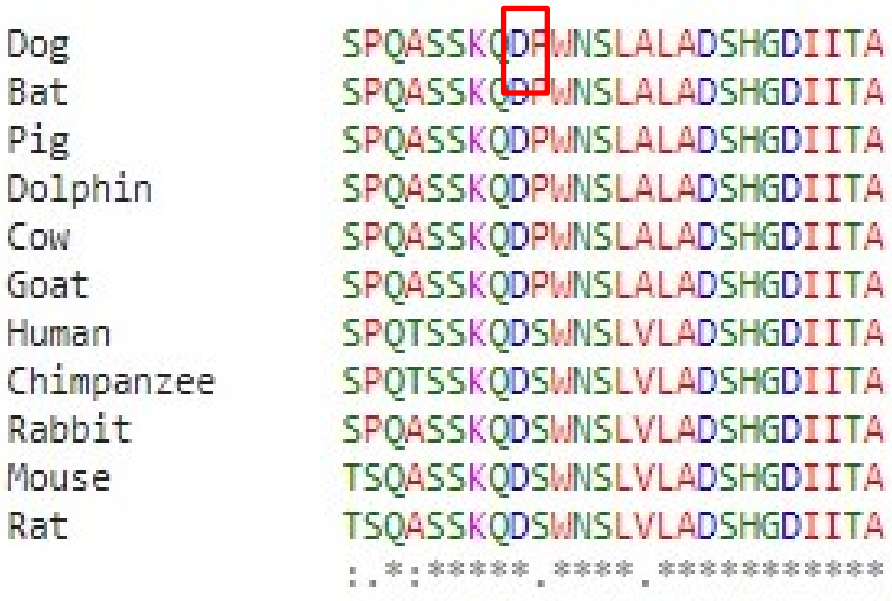


Figure 6-2. Alignment of multiple GATA4 protein sequences across various species. The altered aminoacid of D425N variant is completely evolutionarily conserved among the examined species.

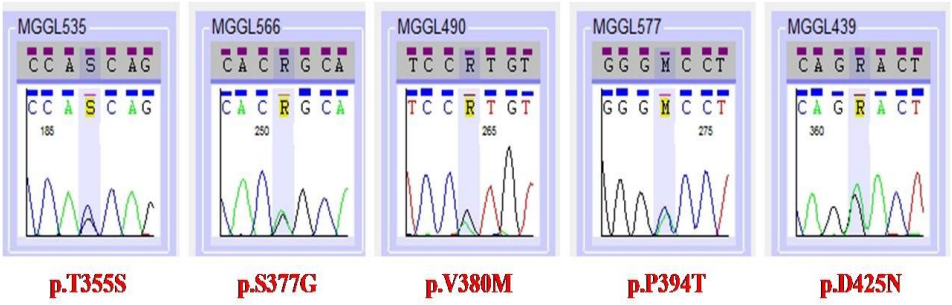
Mutation prediction results showed that the p.T355S, p.S377G, p.V380M and p.P394T variants were predicted to be benign, tolerated and polymorphism by PolyPhen-2, SIFT and

MutationTaster. Only p.D425N variant was predicted to be probably damaging, tolerated and disease causing (Table 6-3).

Table 6-3 Exonic variants in *GATA4* gene coding region

Exon	Nucleotide change	Aminoacid change	dbSNP id	Mutation type	Mutation Prediction		
					Polyphen-2	SIFT	Mutation Taster
5	c.1064C>G	T355S	rs200167770	Missense	Benign	Tolerated	Polymorphism
5	c.1129A>G	S377G	rs3729856	Missense	Benign	Tolerated	Polymorphism
5	c.1138G>A	V380M	rs114868912	Missense	Benign	Tolerated	Polymorphism
6	c.1180C>A	P394T	rs200319078	Missense	Benign	Tolerated	Polymorphism
6	c.1273G>A	D425N	rs56208331	Missense	Probably damaging	Tolerated	Disease causing

A



B

Figure 6-3. (A) Sequence chromatogram indicating five heterozygous missense mutation (p.T355S, p.S377G, p.V380M, p.P394T and p.D425N). (B) Sequence chromatogram showing 15 novel intronic variants.

Table 6-4. (A) Exonic and Intronic variants of *GATA4* gene in CHD patients

	Gender	Sample Type	76885	83271	83502	85342	86268	87409	87813			
1	F	ASD	g.76885T>C	g.83271C>A								
2	F	ASD	g.76885T>C	g.83271C>A								
3	F	ASD, PFO	g.76885T>C	g.83271C>A								
4	M	ASD	g.76885T>C	g.83271C>A	g.83502A>M				g.87813A>W			
5	F	ASD	g.76885T>C	g.83271C>A	g.83502A>M				g.87813A>W			
6	M	ASD	g.76885T>C	g.83271C>A	g.83502A>M				g.87813A>W			
7	F	ASD	g.76885T>C	g.83271C>A	g.83502A>M				g.87813A>W			
8	F	ASD	g. 76885T>C	g.83271C>A	g.83502A>M				g.87813A>W			
9	F	ASD	g.76885T>C	g.83271C>A	g.83502A>M				g.87813A>W			
10	F	VSD	g.76885T>C	g.83271C>A	g.83502A>M				g.87813A>W			
11	M	VSD	g.76885T>C	g.83271C>A	g.83502A>M				g.87813A>W			
12	M	ASD, PDA	g.76885T>C	g.83271C>A	g.83502A>M				g.87813A>W			
13	F	ASD, PDA	g.76885T>C	g.83271C>A	g.83502A>M				g.87813A>W			
14	F	ASD, PDA	g.76885T>C	g.83271C>A	g.83502A>M				g.87813A>W			
15	F	PFO	g.76885T>C	g.83271C>A	g.83502A>M				g.87813A>W			
16	F	ASD,VSD	g.76885T>C	g.83271C>A	g.83502A>M				g.87813A>W	g.76937G>S		
17	M	ASD	g.76885T>C	g.83271C>A	g.83502A>C				g.87813A>W			
18	F	ASD	g.76885T>C	g.83271C>A	g.83502A>C				g.87813A>W			
19	M	ASD, PDA	g.76885T>C	g.83271C>A	g.83502A>C				g.87813A>W			
20	M	ASD,VSD	g.76885T>C	g.83271C>A	g.83502A>C				g.87813A>T			
21	M	VSD,TOF	g.76885T>C	g.83271C>A	g.83502A>C				g.87813A>T			
22	F	ASD	g.76885T>C	g.83271C>A	g.83502A>C				g.87813A>T			
23	NIL	ASD	g.76885T>C	g.83271C>A	g.83502A>C				g.87813A>T			
24	M	ASD,PDA	g.76885T>C	g.83271C>A					g.87813A>W	g.78343G>R		
25	F	ASD, VSD, PDA	g.76885T>C	g.83271C>A	g.83502A>M				g.87813A>W		g.85294C>Y	
26	M	ASD	g.76885T>C	g.83271C>A	g.83502A>M				g.87813A>W	p.T355S	g.84991G>R	
58	M	VSD	g.76885T>C	g.83271C>A					g.87813A>W		p.P394T	g.84991G>R
59	F	VSD	g.76885T>C	g.83271C>A	g.83502A>M				g.87813A>W		p.P394T	
27	M	ASD, PDA	g.76885T>C	g.83271C>A	g.83502A>M				g.87813A>W	p.T355S		
28	F	ASD	g.76885T>C	g.83271C>A		g.85342C>Y	g.86268A>R	g.87409G>R				
29	F	VSD	g.76885T>C	g.83271C>A		g.85342C>Y	g.86268A>R	g.87409G>R				
30	F	PDA	g.76885T>C	g.83271C>A		g.85342C>Y	g.86268A>R	g.87409G>R				
31	M	ASD,PDA	g.76885T>C	g.83271C>A		g.85342C>Y	g.86268A>R	g.87409G>R				
32	NIL	ASD,VSD,PDA	g.76885T>C	g.83271C>A	g.83502A>M	g.85342C>Y	g.86268A>R	g.87409G>R				
33	F	ASD	g.76885T>C	g.83271C>A		g.85342C>Y	g.86268A>R	g.87409G>A				
34	F	ASD	g.76885T>C	g.83271C>A		g.85342C>Y	g.86268A>R	g.87409G>R			g.85294C>Y	p.V380M
35	M	ASD	g.76885T>Y	g.83271C>M		g.85342C>Y	g.86268A>R	g.87409G>R		g.83415G>R	g.85294C>Y	p.V380M
36	M	VSD	g.76885T>C	g.83271C>A		g.85342C>T		g.87409G>A				
37	M	ASD	g.76885T>C	g.83271C>A		g.85342C>T		g.87409G>A				
38	M	ASD,PDA	g.76885T>C	g.83271C>A			g.86268A>R	g.87409G>R			p. P394T	
39	NIL	ASD, VSD	g.76885T>C			g.85342C>Y	g.86268A>G	g.87409G>R	g.87813A>W	g.76937G>S	p.S377G	
40	M	ASD,VSD	g.76885T>C	g.83271C>A	g.83502A>M	g.85342C>Y	g.86268A>R	g.87409G>R	g.87813A>W			
41	M	PDA	g.76885T>C	g.83271C>A	g.83502A>M	g.85342C>Y	g.86268A>R	g.87409G>R	g.87813A>W			
42	M	PDA	g.76885T>C	g.83271C>A	g.83502A>M	g.85342C>Y	g.86268A>R	g.87409G>R	g.87813A>W			
43	M	ASD	g.76885T>C	g.83271C>A	g.83502A>M	g.85342C>Y	g.86268A>R	g.87409G>R	g.87813A>W			
44	F	ASD	g.76885T>C	g.83271C>A	g.83502A>M	g.85342C>Y	g.86268A>R	g.87409G>R	g.87813A>W		g.83318G>K	
45	F	VSD	g.76885T>Y	g.83271C>M	g.83502A>M	g.85342C>Y	g.86268A>R	g.87409G>R	g.87813A>W		g.83415G>R	
46	F	VSD	g.76885T>Y	g.83271C>M	g.83502A>M	g.85342C>Y	g.86268A>R	g.87409G>R	g.87813A>T		g.83415G>R	
47	F	VSD	g.76885T>C	g.83271C>A	g.83502A>M	g.85342C>Y	g.86268A>R	g.87409G>R	g.87813A>W			
48	M	PS	g.76885T>C	g.83271C>A	g.83502A>M	g.85342C>Y	g.86268A>R	g.87409G>R	g.87813A>W			
49	M	ASD,PDA	g.76885T>Y	g.83271C>M		g.85342C>Y	g.86268A>R	g.87409G>R	g.87813A>T			
50	M	ASD, VSD, PDA	g.76885T>C	g.83271C>A	g.83502A>M	g.85342C>Y	g.86268A>R	g.87409G>R	g.87813A>W		g.87725T>Y	
51	M	VSD, DORV	g.76885T>C	g.83271C>M			g.86268A>R		g.87813A>W	g.78343G>R	g.76937G>S	g.83073T>Y
52	F	TOF, VSD, PS	g.76885T>C	g.83271C>M	g.83502A>M		g.86268A>R		g.87813A>T	g.76937G>S	p.S377G	
53	F	ASD,VSD,PDA	g.76885T>Y		g.83502A>M		g.86268A>R		g.87813A>W		p. D425N	
54	M	VSD	g.76885T>C	g.83271C>M	g.83502A>M		g.86268A>R		g.87813A>T	g.76937G>S	p.S377G	
56	F	ASD	g.76885T>C	g.83271C>M	g.83502A>M		g.86268A>R		g.87813A>W	g.76937G>S	p.D425N	

Table 6-4. (B) Exonic and Intronic variants of *GATA4* gene in control group

	Gender	Sample Type	76885	83271	83502	85342	86268	87409	87813	
1	F	Control1	g.76885T>C	g.83271C>A						
2	M	Control2	g.76885T>C	g.83271C>A	g.83502A>C					
3	F	Control3	g.76885T>C	g.83271C>A	g.83502A>M					
4	Nil	Control4	g.76885T>C		g.76937G>S		g.86268A>R			p.S377G
5	F	Control5	g.76885T>C	g.83271C>A		g.85342C>T	g.86268A>G			
6	M	Control6	g.76885T>C	g.83271C>A	g.83502A>M		g.86268A>R	g.87409G>R	g.87813A>T	
7	M	Control7	g.76885T>C	g.83271C>A			g.86268A>R	g.87409G>R		
8	M	Control8	g.76885T>C	g.83271C>A	g.83502A>M	g.85342C>Y	g.86268A>R	g.87409G>R	g.87813A>W	
9	F	Control9	g.76885T>C	g.83271C>A	g.83502A>M	g.85342C>Y	g.86268A>R	g.87409G>R	g.87813A>T	g.78042G>R
10	Nil	Control10	g.76885T>Y	g.83271C>M	g.83502A>M	g.85342C>Y	g.86268A>R	g.87409G>R	g.87813A>W	g.83415G>R

Table 6-5 Genotypic pattern of *GATA4* gene at 85342, 86268, 87409 and 87813 positions in CHD patients (6-5 A) and control group (6-5 B)

6-5 A: CHD patients

S.No.	Frequency	85342	86268	87409	87813
*1.	3	CC	AA	GG	AA
2.	22	CC	AA	GG	AT
3.	4	CC	AA	GG	TT
4.	7	CT	AG	AG	AA
5.	1	CT	AG	AA	AA
6.	2	TT	AA	AA	AA
7.	1	CC	AG	AG	AA
*8.	11	CT	AG	AG	AT
9.	1	CT	GG	AG	AT
10.	3	CC	AG	GG	AT
11.	2	CC	AG	GG	TT
12.	1	CC	GG	GG	TT
13.	1	CT	AA	AG	AT
14.	1	CC	AG	GG	AA

6-5 B: Control group

S.No.	Frequency	85342	86268	87409	87813
*1.	3	CC	AA	GG	AA
2.	1	CC	AG	GG	AA
3.	1	TT	GG	GG	AA
4.	1	CC	AG	AG	TT
5.	1	CC	AG	AG	AA
*6.	2	CT	AG	AG	AT
7.	1	CT	AG	AG	TT

* Genotypic patterns are identical in both CHD patients and control group

Apart from these missense variants of *GATA4* gene, there were 15 novel intronic variants found in different CHD phenotypes as shown in Figure 6-3 B. These intronic variants were analyzed for potential branch point and potential ISEs and ISSs. The results indicated two variants predicted which could be potential branch point sites. One potential branch point site at g.83271C>A/M variant with GCCTAAG motif of 83.82 consensus value in wild type and in addition, this mutation leads to formation of ISS motif 2 (AGAGTGCA) with 63.85 consensus value (Figure 6-4 A) and another potential branch point site at g.86268A>R variant with GGCTGAA motif of 82.24 consensus value in wild type. These intronic mutations at the potential branch point are predicted to be disrupted (Figure 6-4 B).

Heptamer position	Heptamer cDNA position	Branch Point position	Branch Point cDNA position	Branch Point motif	Consensus value (0-100)
44	+44	49	+49	CCCTCAG	97.27
46	+46	51	+51	CTCAGAG	73.89
54	+54	59	+59	GCCTAAG	83.82
117	+117	122	+122	ATCGGAC	72.36
133	+133	138	+138	CTTTCAG	70.07
152	+152	157	+157	AGCCCAG	79.41
206	+206	211	+211	GGCTCAA	87.17
211	+211	216	+216	AACTCAA	82.27
224	+224	229	+229	GCCTGAT	89.54
234	+234	239	+239	TGCCGAC	79.93
247	+247	252	+252	GACCCAG	79.74
254	+254	259	+259	TGCTCAG	91.17
269	+269	274	+274	AGCCGAG	74.48

A

Heptamer position	Heptamer cDNA position	Branch Point position	Branch Point cDNA position	Branch Point motif	Consensus value (0-100)
1002	+1002	1007	+1007	TGCTCAG	91.17
1013	+1013	1018	+1018	AGCTGAT	82.34
1020	+1020	1025	+1025	GCATCAC	72.65
1024	+1024	1029	+1029	CACCCAG	81.37
1026	+1026	1031	+1031	CCCAGAC	79.98
1033	+1033	1038	+1038	CCTTCAT	72.27
1039	+1039	1044	+1044	TGCCTAG	70.31
1091	+1091	1096	+1096	TCCTGAG	90.81
1098	+1098	1103	+1103	GGCTGAA	82.24
1136	+1136	1141	+1141	CCCTCCC	70.37
1139	+1139	1144	+1144	TCCCCAG	86.72
1153	+1153	1158	+1158	CTCCCAA	80.98
1154	+1154	1159	+1159	TCCCAAG	74.89
1161	+1161	1166	+1166	CCCTCAG	97.27
1182	+1182	1187	+1187	GCCTCAT	94.48

B

Figure 6-4. *In silico* analysis of intronic variants by HSF3.0. (A) Potential branch point showing consensus value of g.83271C>A/M variant (B) Potential branch point showing consensus value of g.86268A>R variant.

The g.76885T>C/Y intronic variant leads to formation of new ISE motif for SRp40; whereas the g.76937G>S and g.83073T>C/Y variants lead to the disruption of SRp40 and SRp55 ISE motifs respectively. On the other hand, g.78343G>A/R variant leads to the disruption of ISS motif 1 (CAAAACGT) and g.87813A>T/W leads to formation of ISS motif 2. The g.83318G>K variant leads to the formation of SF2/ASF ISE motif and ISS Motif 2, whereas g.85294C>Y variant leads to the disruption of SRp40 ISE motif and formation of ISS motif 2. The g.84991G>R variant leads to the disruption of ISS motif 1 and formation of ISS motif 2 and the g.87725T>Y variant leads to the formation of SF2/ASF ISE motif and ISS motif 1.

The g.83415G>R variant leads to formation of SRp40 ISE motif and formation of ISS motif 3 and disruption of SF2/ASF2 ISE motif; g.85342C>T/Y leads to formation of SRp55 ISE motif and formation of ISS motif 2. The g.87409G>A/R leads to formation of SRp40 ISE motif and disruption of ISS motif 1. However, intronic variant g.83502A>C/M revealed no significance to the formation of potential branch point site or change of ISE and ISS (Table 6-6).

Intron	Intronic mutations	Mutation frequency in subjects	Branch Point Analysis					
			Potential branch point		Enhancer motifs		Silencer motifs	
			Wild type	Mutant type	Wild type	Mutant type	Wild type	Mutant type
1	g.76885T>C g.76885T>Y	55/60 5/60	-	-	-	Formation of SRp40 binding motif 81.26 (78.08)	-	-
1	g.76937G>C g.76937G>S	2/60 7/60	-	-	SRp40 binding motif 79.70 (78.08)	Disruption of SRp40 binding motif	-	-
3	g.78343G>R	2/60	-	-	-	-	Motif 1 61.17 (60)	Disruption of Motif 1
3	g.83073T>Y	1/60	-	-	SRp55 binding motif 77.0(73.86)	Disruption of SRp55 binding motif	-	-

4	g.83271C>A g.83271C>M	48/60 10/60	**GCCTAAG 83.82 (67)**	Disruption of GCCTAAG site	-	-	-	Formation of Motif 2 63.85 (60)
4	g.83318G>K	1/60	-	-	-	Formation of SF2/ASF binding motif 77.15 (72.98)	-	Formation of Motif 2 70.71 (60)
4	g.83415G>R	3/60	-	-	SF2/ASF2 binding motif 72.15 (72.98)	Disruption of SF2/ASF2 & Formation of SRp40 binding motifs 85.51 (78.08)	-	Formation of Motif 3 63.50 (60)
4	g.83502A>C g.83502A>M	7/60 33/60	No significant mutation prediction					
4	g.84991G>R	2/60	-	-	-	-	Motif 1 70.15 (60)	Disruption of Motif 1 & Formation of Motif 2 66.40 (60)
5	g.85294C>Y	3/60	-	-	SRp40 binding motif 84.97(78.08)	Disruption of SRp40 binding motif	-	Formation of Motif 2 62.75 (60)
5	g.85342C>T g.85342C>Y	2/60 21/60	GCCGCAC 83.28 (67)	GCCGCAC – 79.39	-	Formation of SRp55 binding motif 77.19 (73.86)	-	Formation of Motif 2 68.15 (60)
5	g.86268A>G g.86268A>R	2/60 26/60	**GGCTGAA 82.24 (67)**	Disruption of GGCTGAA site	-	-	-	-
6	g.87409G>A g.87409G>R	3/60 21/60	-	-	-	Formation of SRp40 binding motif 78.98 (78.08)	Motif 1 65.06 (60)	Disruption of Motif 1
6	g.87725T>Y	1/60	-	-	-	Formation of SF2/ASF binding motif 92.18 (72.98)	-	Formation of Motif 1 72.64 (60)
6	g.87813A>T g.87813A>W	9/60 36/60	-	-	-	-	-	Formation of Motif 2 61.41 (60)

Table 6-6 Branch point analysis showing Potential branch points, Enhancer motifs (ISE) and Silencer motifs (ISS) for fifteen intronic variants

* Threshold value of ISEs and ISSs motif values are given in parentheses

***NKX2-5* gene analysis**

Direct sequencing of *NKX2-5* gene yielded three known synonymous variants c.63A>G (p.Glu21Glu), c.180G>A (p.Glu60Glu) and c.606G>C (p.Leu202Leu) and one known nonsynonymous variant c.298C>G (p.Pro100Ala) in exon 1 and exon 2 regions respectively. Their dbSNP ids were rs2277923, rs373636712 and rs3729753 for synonymous variants and rs550046293 for nonsynonymous variant (Figure 6-5).

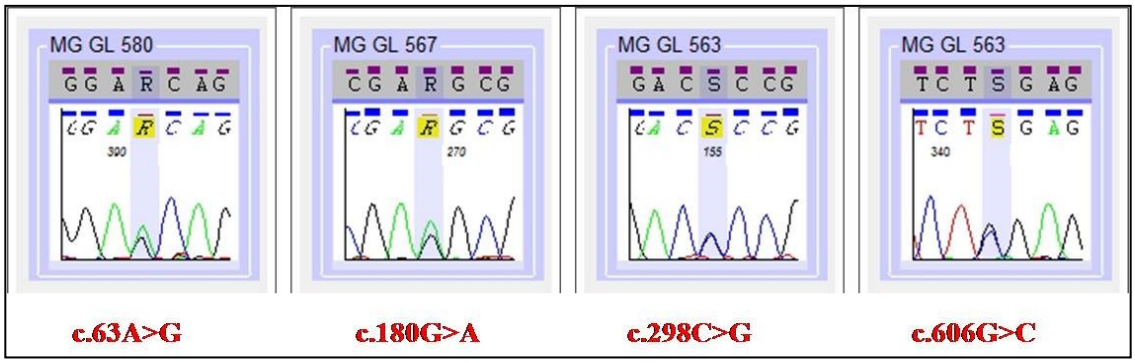


Figure 6-5. Sequence chromatogram showing three synonymous variants (c.63A>G, c.180G>A and c.606G>C) and one nonsynonymous variant (c.298C>G) in *NKX2-5* gene coding region.

In this study population, frequency of the synonymous variant p.Glu21Glu were found in 34 patients (56.7%) and among them 23 patients were with heterozygous condition (g.5292A>R). Two patients (3.3%) were with p.Glu60Glu variant and one patient (1.67%) with nonsynonymous variant (p.Pro100Ala) and eight patients (13.3%) with heterozygous condition of p.Leu202Leu variant. All these variants were identified in various clinical conditions of 36 subjects as listed in Table 6-7 A & B, while other 24 patients did not show up any sequence variant.

Table 6-7 (A). Frequency of *NKX2-5* gene sequence variants identified in different types of CHDs.

c.63A>G		c.180G>A		c.298C>G		c.606G>C	
CHD	**Frequency in subjects (n=60)**	**CHD**	**Frequency in subjects (n=60)**	**CHD**	**Frequency in subjects (n=60)**	**CHD**	**Frequency in subjects (n=60)**
ASD	10	TOF	1	ASD,PDA	1	ASD	1
VSD	6	ASD,VSD	1			PDA	2
PDA	3					ASD,PDA	4
PS	1					ASD,VSD	1
TOF	1						
ASD, PDA	7						
ASD, VSD	2						
ASD,VSD,PDA	3						
DORV	1						

Table 6-7 (B). Types of CHDs and sequence variants observed in *NKX2-5* gene in the study group.

S. No	Sex	Types of Congenital Heart Diseases	*NKX2-5* gene sequence variants
1	M	ASD (Ostium secundum)	c.63A>G
2	M	ASD (Ostium secundum)	c.63A>G
3	NIL	ASD (Ostium secundum)	c.63A>G
4	F	ASD (Ostium secundum)	c.63A>G, c.606G>C
5	F	ASD (Ostium secundum)	c.63A>G
6	F	ASD (Ostium secundum)	c.63A>G
7	M	ASD (Ostium secundum)	c.63A>G
8	F	ASD (Ostium secundum)	c.63A>G
9	M	VSD (Muscular)	c.63A>G
10	F	VSD (Perimembranous)	c.63A>G
11	F	VSD (Perimembranous)	c.63A>G
12	F	VSD (Perimembranous)	c.63A>G
13	F	VSD (Perimembranous)	c.63A>G
14	F	VSD (Perimembranous)	c.63A>G

15	M	Patent Ductus Arteriosus	c.63A>G
16	F	Patent Ductus Arteriosus	c.63A>G, c.606G>C
17	M	Patent Ductus Arteriosus	c.63A>G, c.606G>C
18	M	Valvar Pulmonary Stenosis	c.63A>G
19	M	TOF (Large inlet perimembranous VSD)	c.63A>G, c.180G>A
20	F	ASD (Ostium secundum)	c.63A>G
21	F	ASD (Ostium Primum) VSD (Perimembranous)	c.63A>G, c.180G>A
22	M	ASD (Ostium secundum) VSD (Upper muscular)	c.606G>C
23	NIL	ASD (Ostium secundum) VSD (Perimembranous)	c.63A>G
24	F	ASD (Ostium secundum) Patent Ductus Arteriosus	c.63A>G, c.606G>C
25	M	ASD (Ostium secundum) Patent Ductus Arteriosus	c.63A>G
26	M	ASD (Ostium secundum) Patent Ductus Arteriosus	c.63A>G
27	M	ASD (Ostium secundum) Patent Ductus Arteriosus	c.63A>G, c.606G>C
28	M	ASD (Ostium secundum) Patent Ductus Arteriosus	c.63A>G, c.298C>G, c.606G>C
29	M	ASD (Ostium secundum) Patent Ductus Arteriosus	c.63A>G
30	F	ASD (Ostium secundum) Patent Ductus Arteriosus	c.63A>G
31	M	ASD (Ostium secundum) Patent Ductus Arteriosus	c.606G>C
32	F	ASD (Ostium secundum)	c.63A>G
33	M	DORV (Subaortic VSD)	c.63A>G
34	F	ASD (Ostium secundum) VSD (Perimembranous) Patent Ductus Arteriosus	c.63A>G
35	M	ASD (Ostium secundum) VSD (Perimembranous) Patent Ductus Arteriosus	c.63A>G
36	F	ASD (Ostium secundum) VSD (Perimembranous) Patent Ductus Arteriosus	c.63A>G

Mutation prediction results showed that the p.Glu21Glu, p.Pro100Ala and p.Leu202Leu variants as tolerated and non-disease causing by SIFT and polymorphism by MutationTaster. Only p.Glu60Glu variant (synonymous SNP) could be predicted as tolerated but disease causing, with

the SIFT score of 0.43 and probability value of 0.999, providing evidence as disease causing variant rather than a benign polymorphism Table 6-8. This variant was seen in two patients in our study group, one with TOF and another patient with ASD and VSD. The presence of nonsynonymous SNP (c.298C>G) in this study was seen in a patient with ASD and PDA condition, along with the two common SNPs (c.63A>G, c.606G>C).

Table 6-8. Exonic variants identified in *NKX2-5* gene using SIFT and MutationTaster prediction tools.

Exon	Nucleotide change	Aminoacid change	dbSNP id	SNP Type	Mutation Prediction	
					SIFT	MutationTaster
1	c.63A>G	p.Glu21Glu	rs2277923	Synonymous	Tolerated	Polymorphism
1	c.180G>A	p.Glu60Glu	rs373636712	Synonymous	Tolerated	Disease causing
1	c.298C>G	p.Pro100Ala	rs550046293	Nonsynonymous	Tolerated	Polymorphism
2	c.606G>C	p.Leu202Leu	rs3729753	Synonymous	Tolerated	Polymorphism

Discussion

Pre-mRNA splicing is critical in the regulation of gene expression and is carried out by a large ribonucleoprotein complex called the spliceosome, which includes the five major snRNPs, U1, U2, U4, U5, and U6 (236). Regulation of transcription and RNA processing is complex, and increasing evidence suggests that transcription and splicing may be coordinated through the action of several proteins (237). Pre-mRNA splicing is an essential process in gene expression and majority of human genes undergo alternative splicing, leading to the formation of isoforms (236). It is regulated by *cis*-elements (ESE, ESS, ISE and ISS) and *trans*-acting factors such as Serine and Arginine-rich proteins (SR proteins) and heterogeneous nuclear ribonucleoprotein (hnRNP) (238).

Intronic variants and its consequence on the splicing mechanism can be better understood by using *in silico* analysis that includes different algorithms to identify various regulatory elements. *In silico* analysis (HSF3.0) showed that the intronic variant g.86268A>R predicted as potential branch point site. Consensus wild type score for the potential branch point ranges from 0 to 100 and the threshold value is defined to be 67. Scores above 67 is considered to be potential branch point. When a mutation occurs, if the wild type score is above 67 and the score variation (between wild type and mutant) decreases by more than 10%, it is considered that the mutation breaks the branch point. In the intronic variant, g.83271C>A/M apart from the loss of potential branch point, it also lead to the formation of ISS motif 2. Alterations in the branch point position might provoke serious defects in pre-mRNA splicing leading to exon skipping or intron inclusion or activation of cryptic splice sites (239-241).

ESEs/ESSs will promote or inhibit inclusion of exon, where as ISEs/ISSs will enhance or inhibit adjacent splice sites usage (238). ESEs/ISEs are characterized as small RNA motifs that act as binding sites for SR proteins to enhance splicing by spliceosome recruitment (242). ESSs/ISSs will bind to negative regulators including hnRNP family to oppose the positive effect of SR proteins and repress splicing (243-245). SR proteins are RNA-binding proteins consists of C-terminal domain with highly conserved amino acid sequences (RS domain) and N-terminal RNA recognition domain (RRM domain) (246). Presently, nine human SR proteins have been identified (247) and its characteristic feature is the capability to regulate 5' splice site choice (246). Among SR proteins, SF2/ASF, SRp40 and SRp55 were well-studied in alternative splicing. SR proteins act as splicing activators and can cooperate and compete together in splicing regulation (247).

SF2/ASF (SRSF1) was the first identified and most abundant protein which transfers between the nucleus and cytoplasm (248) and its consensus sequence is SRSASGA (S indicates G/C, R indicates Purine), rich with purine residues (247). SRp40 (SRSF5) and SRp55 (SRSF6) are characterized with C-terminal (RS domain), RRM domain and RRM homology domain (249). SRp40 yields the consensus sequence ACDGS (D indicates other than C, S indicates G/C) and SRp55 has consensus sequences USCGKM (S indicates G/C, K indicates U/G, M indicates A/C) (247,249). SRp40 ESEs/ISEs are also activated by SRp55 because they are closely related in domain structures and molecular mass of 31.2 kD, 39.6 kD and 65% sequence identity (247). At higher concentrations of SF2/ASF, it will help to use the sites which are proximal to 3' splice site, whereas SRp40 and SRp55 will stimulate to use of distal sites and further expands the association of U1 snRNP with distal sites (246).

ISEs/ISSs are with primary importance in splicing regulation, due to the conserved sequences surrounding constitutive exons, around a distance of 150 bp or more in mammals (250). Among ISSs, motif 2 – (U/G)G(U/A)GGGG proved as a strong splicing silencer than motif 1(CTAGAGGT) and motif 3 (TCTCCCAA), where it predicted to wield too weak silencing effects for making any variation in splicing (242). Another two groups identified Fox-1/2 binding motif (UGCAUG) with more conserved sequence, associated with tissue-specific splicing factors (251,252).

The cause of the CHD phenotypes seem to be mainly due to the disruption of branch point sites either with homozygous condition at g.83271C>A position or with heterozygous condition at g.83271C>M position along with g.86268A>R position. The consensus sequences with respect to these branch point positions have been illustrated in (Figure 6-6).

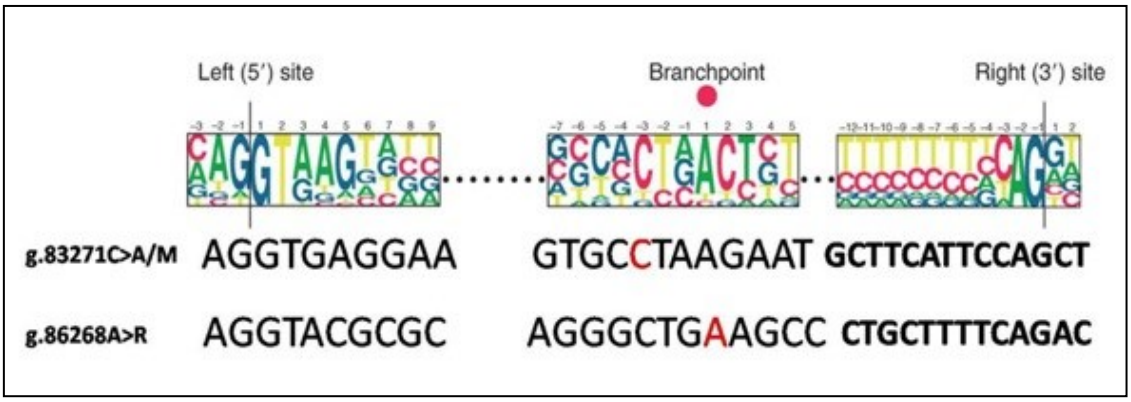


Figure 6-6. Consensus sequence showing potential branch point sites (red color) at g.83271C>A/M position and at g.86268A>R position.

The variants g.85342C>T/Y, g.86268A>G/R, g.87409G>A/R and g.87813A>T/W were observed in both cases and controls. Interestingly, these genotypic patterns observed in cases were not identical with the controls except two patterns as represented in Table 6-5. This observation suggested the possibility of other intronic variants presented responsible for the development of CHD. The other variants namely g.76937G>S, g.78343G>A/R and g.83073T>C/Y were seen in DORV condition and in PDA, apart from g.76885T>C/Y, g.83271C>A/M, g.86268A>R, and g.87813A>T/W variants, the g.87409G>A/R was picked up consistently in all cases. In one VSD, there were two missense heterozygous mutations (p.T355S and p.S377G) along with other intronic variants. To the best of our knowledge, all these potential branch point mutations, mutations in ISEs and ISSs have not been presented in the literature. In the present study, we could not establish the role of these intronic variants to different clinical condition of CHDs and thus it could be attributed essentially to mutations in other transcription factors.

All the sequence variants obtained were registered in Clinvar and their dbSNP ids are listed in the Table 6-9. ClinVar is a freely accessible, public archive of reports of the relationships among human genetic variations and phenotypes, with supporting evidence. It facilitates access to and

communication about the relationships asserted between human variation and observed health status, and the history of that interpretation. ClinVar processes submissions reporting variants found in patient samples, assertions made regarding their clinical significance, information about the submitter, and other supporting data. The alleles described in submissions are mapped to reference sequences, and reported according to the HGVS standard. It contains information about genomic variations and their relationships to human health. It finds genomic variants and the diseases for which the variant or set of variants was interpreted. It will also find phenotypes reported for individuals with the variant and to examine reported clinical significance of each variant and assess information on quality and consistency of interpretations of each variants effect.

Table 6-9. *GATA4* Sequence variants and their dbSNP ids from Clinvar database.

Intronic variant	Location	dbSNP id
g.76885T>C	NM_002052.4(GATA4):c.617-116T>C	**rs3735819**
g.76937G>C	NM_002052.4(GATA4):c.617-64G>C	**rs10503425**
g.78343G>A	NM_002052.4(GATA4):c.909+25G>A	**rs147860174**
g.83271C>A	NM_002052.4(GATA4):c.997+56C>A	**rs804280**
g.83318G>T	NM_002052.4(GATA4):c.997+103G>T	**rs113049875**
g.83415G>A	NM_002052.4(GATA4):c.997+200G>A	**rs3729851**
g.85294C>T	NM_002052.4(GATA4):c.1146+129C>T	**rs116052854**
g.85342C>T	NM_002052.4(GATA4):c.1146+177C>T	**rs12156163**
g.86268A>G	NM_002052.4(GATA4):c.1147-107A>G	**rs745379**
g.87409G>A	NM_002052.4(GATA4):c.*852G>A	**rs804290**
g.87725T>C	NM_002052.4(GATA4):c.*1168T>C	**rs549543886**
g.87813A>T	NM_002052.4(GATA4):c.*1256A>T	**rs12458**

NKX2-5 gene interacts through the homeodomain physically and synergistically with *TBX5* and *GATA4* genes and disruptions in this mechanisms lead to CHD. Sequence variants identified in this homeodomain has been proved for less interaction with *TBX5* and *GATA4* genes (148). Several studies have identified both germline and sporadic *NKX2-5* mutations responsible for the CHD malformation and these mutations are mainly reported in homeodomain region followed by TN and NK2-SD domains and also one in splice-site junction (148,253). Mutations associated with

HD and NK2-SD domain lead to truncated protein and these regions play an essential role for the construction of the conduction system (120). Mutations in HD domain particularly have been shown to either loss or reduced DNA binding, transactivation activities and protein-protein interactions (150).

Studies from different countries have proved the association between cardiac septal defects and *NKX2-5* gene mutations, whereas there are very few reports on *NKX2-5* sequence variants to prove the genotype-phenotype correlation among Indian CHD patients (151,152). The available reports are on the known *NKX2-5* sequence variants and its association with respect to CHD condition. The absence of *NKX2-5* mutation in a study group lead to the suggestion that exon 1 region might not be implicated in CHD condition and proposed for a mosaic nature (152). The common SNP (c.239A>G) was seen both in cases and control group and due to this neutral effect of synonymous SNP (Glu21Glu), it was not implicated for the disease manifestation. They also had identified 1212G>T SNP in 3'UTR region in 40% of CHD cases (151).

In sequencing analysis of *NKX2-5* gene, 4 sequence variants were identified in 60 CHD patients and among them c.63A>G (rs2277923) located in the exon 1 and c.606G>C (rs3729753) located in the exon 2 and all were known common SNPs reported in the literature (254). No novel exonic and intronic splice-site mutation was identified in this *NKX2-5* gene. c.63A>G (rs2277923) and c.606G>C (rs3729753) were two well studied SNPs among CHD. Meta-analysis revealed heterogeneity of SNPs both in c.63A>G (rs2277923) as well as c.606G>C (rs3729753) and therefore were not implicated in the pathogenesis of CHD (148,254). Further, it has also been stated that synonymous SNP do not change the amino acid sequence, it might affect mRNA structure and stability, affecting gene expression, protein synthesis and function (254).

However, extensive mutational analysis of *NKX2-5* gene for sequence variants in exonic and intron-exon boundary regions did not yield any such interesting results which got picked up in *GATA4* gene analysis. Though, this study was aimed to look for novel sequence variants in South Indian patients with CHD, this study has reiterated the significance of four sequence variants in different types of CHDs and all these variants were detected even within a small study group. Among the variants, c.63A>G SNP were observed more in ASD, followed by ASD and PDA condition and c.606G>C SNP were seen in ASD and PDA condition. However, in this study no splice-site junction mutations were picked up probably could be attributed to the sample size as the limitation.

Conclusion

In conclusion, this study expands the spectrum of *GATA4* intronic mutations in Indian population and provides additional information on molecular aberration seen in CHD patients. The sequence variants search in *NKX2-5* gene revealed that the variants are restricted to exons 1 and 2, similar to the reports across various population studies and sans the scope of further mutational analysis in finding novel mutations in this gene. This also suggests that *NKX2-5* gene mutations could be conserved and specific. However, further studies in this *GATA4* and *NKX2-5* genes in a larger population might provide additional molecular genetic basis of CHD.

CHAPTER-7

Analysis of magnitude of oxidative DNA damage of the peripheral blood lymphocytes in patients suffering from Congenital Heart Diseases

Introduction

Congenital heart diseases (CHD) are defects in the structure of the heart which are present in the offsprings at their birth. There are two types of CHD usually divided into two groups cyanotic and acyanotic heart defects. In the case of non-cyanotic (acyanotic) heart defects, blood flows from the left side of heart to the right side of the heart due to structural abnormality. Individual with left to right shunting often retain or present with normal oxygenated saturation in systemic circulation causing left to right shunt lesion include ASD, VSD, PDA , ECD and PAPVR. Left to right shunt causes an elevation of pulmonary blood flow, which triggers obstructive and obliterative alteration in the pulmonary vascular bed and a progressive increase in pulmonary vascular tissue (199-202). Congenital Heart Diseases (CHD), either due to septal defect or great vessel anomaly is more prone for hypoxia (43,203). Chronic hypoxia of CHD results in a down-regulation of antioxidant defenses, making cells vulnerable to oxidative damage (205).

Free radicals are reactive compounds that are produced naturally in the body by breaking a chemical bond and keep one electron either through cleavage of radicals or by redox reactions. Reactive oxygen species (ROS) include both free radicals and nonfree radical oxygenated molecules (255). When ROS elevated at higher concentrations, they generate oxidative stress that can damage lipids, proteins and DNA (195). In DNA the guanine residue is more prone to oxidation and form 8-hydroxydeoxyguanosine (8-OHdG). The measurement of the levels of this molecule has been utilized as an index of DNA damage (215). Review of literature on the oxidative damage of DNA in patients suffering from CHD has indicated paucity of data , though there are information available on the levels of antioxidants, oxidative damage end products in patients suffering from cyanotic as well as acyanotic CHDs (208-210). Since the chances of free radical

generation is much higher in CHD patients as explained it was found interesting to investigate oxidative damage of DNA in these patients.

Materials and Methods

Subjects

The patient group comprised 22 ASD, 12 VSD, 3 cases of ASD combined with VSD and another 3 cases of combined ASD, VSD and PDA. The total number of CHD cases was 40. For a comparison 35 age and sex-matched controls were included in this study. Genomic DNA was extracted from 2 ml of peripheral blood sample collected into sterile EDTA vacutainer using standard salting out method (220). DNA concentration and purity was checked by optical density ratios (260/280 nm) using UV-Vis Spectrophotometer and stored it at -20°C until use. Assay of 8-hydroxydeoxyguanosine (8-OHdG) was carried out as detailed (256).

Principle of the assay

This assay is based on the competition between oxidatively damaged guanine species and an 8-OHdG-acetylcholinesterase conjugate for a limited amount of monoclonal antibody. The electric eel of *Electrophorus electricus* contains an avid Acetylcholinesterase (AChE), which has a clover leaf-shaped tertiary structure of triad of tetramers attached to collagen-like structural fibril. This AChE was covalently attached to 8-OHdG as a tracer in this assay and the plate was pre-coated with goat anti-mouse IgG and blocked with a proprietary formulation of proteins. This was followed by the addition of specific antibody to oxidatively damaged guanine of samples/standards and forms a complex binding to the goat polyclonal anti-mouse IgG that has been previously attached to the well. Then the plate was washed to remove all unbound reagents and color developed with substrate (Ellman's reagent). The intensity of the color is proportional to the

amount of oxidative DNA damage tracer bound to the well, which is inversely proportional to the amount of free 8-OHdG present in the well (256).

Measurement of 8-OHdG

Pre-assay preparation was done by using deionized water to prepare working solutions from the ELISA buffer concentrate (10X) and wash buffer concentrate (400X). Genomic DNA samples were converted into single-stranded DNA by incubating the samples at 95° C for 5 minutes followed by snap chill process. The samples were then subjected to digestion using 1 µl of S1 Nuclease (Thermo scientific) with buffer for 2 hours at 37° C and adjusted the pH to 7.5-8.5 using 1M Tris (pH-8). It was further treated with 1 unit of Alkaline Phosphatase (Thermo scientific) with buffer per 300ng of DNA and the whole preparation was incubated at 37° C for 45 minutes, boiled for 10 minutes and placed on ice until use.

ELISA standards were prepared from the stock (300 ng/ml) to make bulk standard (30 ng/ml) and then with the use of ELISA buffer it was serially diluted to make standards (S1 to S8). Tracer and ELISA monoclonal antibody was reconstituted to perform the assay. ELISA was carried out as per the manufacturer's instructions (256). 100 µl of ELISA buffer was added to non-specific binding (NSB) wells and 50 µl to maximum binding (Bo) wells. Then 50 µl of duplicated standards were added to the respective wells, followed by the addition of 50 µl of tracer in NSB, Bo and standards/sample wells. Only 5 µl of tracer was added to total activity (TA) wells. Finally, 50 µl of monoclonal antibody was added to Bo and standards/samples wells.

Plate was covered with plastic film and incubated for 18 hours at 4°C. After incubation, wells were emptied and rinsed five times with wash buffer. Plate was developed by adding 200 µl of Ellman's reagent to each well. 5 µl of tracer was added to TA wells. Then the plate was covered with the plastic film and the optimum development is obtained with the help of orbital shaker to

develop in the dark for 90-120 minutes. The plate was then read at a wavelength of 405 nm. The levels of 8-OHdG was calculated by the following method. The average absorbance readings of the NSB wells and Bo wells and then subtracted the average NSB readings from average Bo readings to get corrected Bo or corrected maximum binding. The %B/Bo values were calculated and obtained standard curve plot %B/Bo for standards using four parameter logistic fit equation.

Statistical Analyses

The data were statistically analyzed by SPSS software version 22. Values were expressed as means ± standard deviation. The normality of the variables was analyzed using the Kolmogorov-Smirnov test and non-parametric test (Mann-Whitney U test) was used to compare the 8-OHdG values of all the groups. P value < 0.05 was considered statistically significant.

Structural Visualization

The crystal structure of 8-oxoguanine DNA complexed to human 8-oxoguanine glycosylase (hOGG1 Protein Data Bank PDB code 2NOH) was derived and utilized as a model for visualization and analysis using PyMOL software version 2.0.4.

Results

The data on the levels of 8-OHdG (ng/ml) in various types of CHDs and controls is represented in the Table 7-1. Comparison of the levels of 8-OHdG with the controls revealed that the patients with combined ASD, VSD and PDA showed significant increase (37.34±3.54, p value < 0.05) in comparison to the control of (21.26±8.09, p value < 0.05). In the isolated case of VSD, a decrease in the levels of 8-OHdG was observed VSD 14.65±5.01, p value < 0.05). Similarly, isolated case of ASD presented almost closer values (20.34±10.17) to the controls (21.26±8.09) with regard to the 8-OHdG levels. Further, the combination of ASD and VSD presented a marginally higher levels of 8-OHdG when comparison to controls, which was not statistically significant.

Figure 7-1 illustrates graphical representation the levels of 8-OHdG levels in the CHD patients and control groups.

Table 7-1. Comparison of 8-OHdG levels in CHDs and control group.

CASES				CONTROLS
ASD (22)	VSD (12)	ASD, VSD (3)	ASD, VSD, PDA (3)	
20.34±10.17	* 14.65±5.01	26.75±7.94	* 37.34±3.54	* 21.26±8.09

Values are expressed as Mean ± SD. * $p < 0.05$

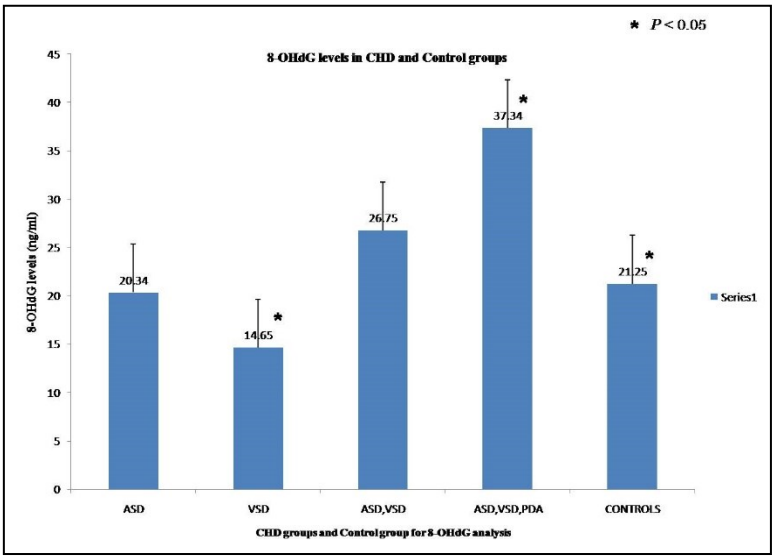
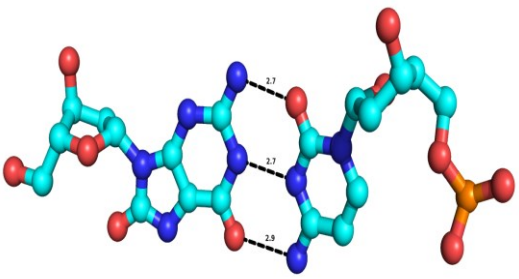


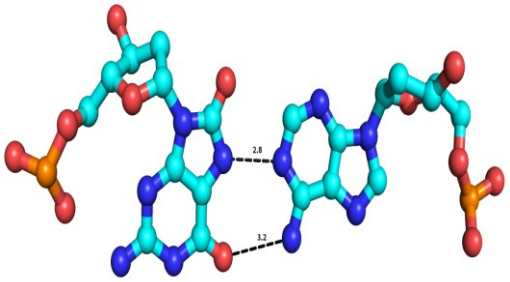
Figure 7-1. 8-OHdG levels (ng/ml) in the CHD patients and control group.

Structural visualization of DNA consequent to 8-OHdG formation and its strucutral analysis using bio-informatics tool showed that 8-OHdG could basepair with cytosine and adenosine residue as pictured in Figure 7-2 (A) and 7-2 (B).



8-Oxo- Cytosin

Figure 7-2. (A) Base pairing of Deoxy-8-Oxo-guanylate with Deoxycytidylate



8-Oxo- Adenosin

Figure 7-2. (B) Base pairing of Deoxy-8-Oxo-guanylate with Deoxyadenylate

Discussion

Oxidative stress is caused by the imbalance between the oxidant and antioxidant systems in the body. Oxidative DNA damage can result from a variety of factors including radiation, toxins, chemicals and reactive oxygen species (40). It is considered to play an important role either as a cause or consequence of several patho-physiological processes (45,257). Oxygen is essential for cardiac viability, function and myocardial gene expression (197). Clinical studies available from the literature on oxidative DNA damage in CHDs have been done by comet assay on cultured lymphocytes and by measurement of biochemical parameters such as glutathione peroxidase, superoxide dismutase, catalase, vitamin E, uric acid, selenium and malondialdehyde (MDA) (205-207). There are not many studies on the oxidative damages on DNA consequent to the patients suffering from various types of CHD particularly with respect to the levels of 8-OHdG. There are studies on the oxidative state of children with cyanotic and acyanotic CHDs and the parameters measured were total antioxidant status, total oxidant status oxidative stress index. The results of these studies indicated that cyanotic group more vulnerable to oxidative stress than the acyanotic group. A comparison between acyanotic (ASD, VSD and PDA) and control groups showed no statistical differences (209). However, in this study the cases were considered discretely (209).

Therefore, an effort was made to measure the levels of 8-OHdG in the following congenital heart diseases conditions such as ASD, VSD, combined ASD,VSD and combination of three deformities ASD,VSD and PDA to ascertain the oxidative damages occurring in DNA molecule in consequence to these disease condition. Results of this study indicated a maximum levels of 8-

OHdG in the case of ASD, VSD and PDA followed by ASD and VSD in comparison to controls. However, the levels of 8-OHdG in ASD and VSD presented a converse result. Comparison of this observation with the study conducted by Ercan *et al.,* on the oxidative status and stress is supportive of the present observation as the oxidative stress / total oxidant status were not that alarming to cause extensive oxidative damage in DNA molecule (209). This might support the reason for near normal 8-OHdG levels observed in ASD and VSD. The salient outcome of this study has been the observation of significantly increased levels of 8-OHdG in combined cases of CHD with the patients presenting 3 conditions ASD, VSD and PDA together. While it is a fact the study had only 3 cases of this condition, the results were encouraging and the need to take up the study with larger number of cases.

Interestingly, the results of this study demonstrated that there was increased DNA damage in combination cases of ASD, VSD with PDA group when compared to that of controls. PDA is reported frequently in preterm infants and ranged from so small size to large enough and cause volume loading of the left ventricle and pulmonary hypertension (258). Studies have shown that there were oxygen sensors in the ductus arteriosus and it is affected by changes in PO_2 (partial presuure of oxygen) and redox state producing ROS (259,260). During birth, ROS increases due to the changes of PO_2 from fetal to neonatal levels (261). Due to this, PDA might generate oxygen radicals causing chronic hypoxia, hypoperfusion and ischemia which exert deleterious effect to manifest the diseased condition (262). The observation of increased 8-OHdG in cases of PDA with septal defects could be partially answered by the mechanism explained as above which affect hemodynamics, redox state and ROS generation.

Conclusion

The analysis of oxidative magnitude of DNA in patients suffering from CHD indicated that near normal values in cases of ASD and VSD. However, significantly increased levels of 8-OHdG were seen in combination cases of ASD, VSD with PDA. The elevated levels of 8-OHdG could be attributed to possible transversion mutations in various genes leading to disturbances in translation process resulting in fatal clinical manifestations.

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Identification of intronic-splice site mutations in *GATA4* gene in Indian patients with congenital heart disease

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ABSTRACT

Congenital Heart Disease (CHD) is the most common birth defect among congenital anomalies that arise before birth. *GATA4* transcription factor plays an important role in foetal heart development. Mutational analysis of *GATA4* gene in CHD patients revealed five known heterozygous mutations (p.T355S, p.S377G, p.V380M, p.P394T and p.D425N) identified in exons 5 and 6 regions and fifteen intronic variants in the non-coding regions (g.76885T > C/Y, g.76937G > S, g.78343G > R, g.83073T > Y, g.83271C > A/M, g.83318G > K, g.83415G > R, g.83502A > C/M, g.84991G > R, g.85294C > Y, g.85342C > T/Y, g.86268A > R, g.87409G > A/R, g.87725T > Y, g.87813A > T/W). *In silico* analysis of these intronic variants identified two potential branch point mutations (g.83271C > A/M, g.86268A > R) and predicted effects of these on intronic splice sites as enhancer and silencer motifs. This study attempts to correlate the pattern of intronic variants of *GATA4* gene which might provide new insights to unravel the possible molecular etiology of CHD.

1. Introduction

Congenital Heart Disease (CHD) is the most common birth defect in humans affecting 1% of all live births in the first year of life and it is one of the major causes of morbidity and mortality in infants [1]. In India, nearly 1,80,000 children are born with CHD with the prevalence ranged from 8 to 10 of every 1000 live births [2]. CHD is a complex multifactorial disorder with genetic and environmental factors playing an important role in disease development [3]. It is categorized mainly into four groups namely septal defects, cyanotic heart disease, obstruction defects and hypoplasia, where genetic factors are implied in the etiology [4]. A group of highly conserved transcription factors such as *GATA4, NKX2-5, MYH6, NOTCH, TBX5, TBX20, ZIC3, TFAP2B* and others are involved in foetal heart development and regulation [5–7]. Among these *GATA4* is well studied and established gene known to cause sporadic and familial non-syndromic CHDs which include atrial septal defect (ASD), ventricular septal defect (VSD), patent ductus arteriosus (PDA), atrioventricular septal defect (AVSD), tetralogy of fallot (TOF) and pulmonary value stenosis (PS) [5–8].

GATA proteins (DNA binding proteins) are involved in cell differentiation, survival and proliferation of tissues that regulate gene expression. They recognize and bind to "*GATA*" consensus sequence of target genes [9]. Six members have been indentified in vertebrates *GATA* family. *GATA1, GATA2,* and *GATA3* are mainly involved in hematopoietic cell expression, whereas *GATA4, GATA5* and *GATA6* are expressed in heart, liver and gonadal tissues [10]. *GATA4* (Gene Id: 2626, OMIM: 600576) is a critical transcription factor expressed in embryo and adult cardiomyocytes and it promotes cardiac morphogenesis, survival and function of the heart [11]. Human *GATA4* gene maps to chromosome 8p23.1- p22 region, a hypermutable protein coding gene encodes 442 amino acids with two transcriptional activation domains (TAD1, 1–74 amino acids; TAD2, 130–177 amino acids), two zinc finger domains (ZF1, 215–240 amino acids; ZF2, 270–294 amino acids) and one nuclear localization signal (NLS, 254–324 amino acids) [12–14]. Mutations associated in *GATA4* gene had been reported in several cardiac diseases such as congenital heart disease, abnormal ventral folding and hypoplasia of ventricular myocardium [15,16]. Till date, more than 120 mutations have been reported in the *GATA4* gene. These mutations in *GATA4* are well-studied and reported from different countries in CHD patients and are implied as reasons for CHD in humans [17].

There are only few studies conducted in Indian population on CHD

Abbreviations: CHD, congenital heart disease; ASD, atrial septal defect; VSD, ventricular septal defect; PDA, patent ductus arteriosus; AVSD, atrioventricular septal defect; TOF, tetralogy of fallot; PS, pulmonary value stenosis; DORV, double outlet right ventricle; TAD, transcriptional activation domain; ZF, zinc finger domain; NLS, nuclear localization signal; EDTA, ethylenediaminetetraacetic acid; PolyPhen-2, polymorphism phenotyping version 2; SIFT, sorting intolerant from tolerant; ESE, exonic splicing enhancer; ESS, exonic splicing silencer; ISE, intronic splicing enhancer; ISS, intronic splicing silencer

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Table 1
List of *GATA4* primers.

Exon	Forward primer (5′–3′)	Reverse Primer (5′–3′)	Annealing temperatue
Exon 2	GAACTCTCAGTGTCTGGGATTAG	GTGGCTCCAGCTAACTCTAAA	61 °C
Exon 3	TGACGGTGAATGATGGTTAGG	GGCCAGCAAAGTAGTTGAAAG	61 °C
Exon 4	CATCACACAGGTGCTCGATAAG	CCAAAGATGAAAGGACCGAGTA	61 °C
Exon 5	TGTAGCCCTCCGCAGATAA	GTCATGTCCACATCACCATCT	61 °C
Exon 6	TTCTGGGCAACCACAGTATC	AGTCCCATCAGCGTGTAAAG	61 °C
Exon 7	GCTCCTTCACTTCCAACATCTC	ACCCTCTCCCAGGAAATTAAAG	61 °C

Table 2
Cardiac septal defects of the subjects.

CHD condition	Number of subjects
ASD	22
VSD	12
PDA	3
ASD, VSD	4
ASD, PDA	9
ASD, VSD, PDA	4
VSD, DORV	1
ASD, PFO	1
VSD, TOF, PS	1
VSD, TOF	1
PS	1
PFO	1
Total Number of Patients	60

to identify the causes of this disease condition [18–20]. The present study was designed to look for mutations in the exonic and intronic regions of *GATA4* gene of patients with CHD.

2. Materials and methods

2.1. Subjects

Seventeen patients diagnosed with isolated CHDs at R. L. Jalappa Hospital and Narayana Hrudalaya heart centre from Kolar were recruited in this study. Pediatric cardiologists confirmed the CHD by two-dimensional echocardiography with color flow doppler. After getting the Institutional Ethics committee approval and written informed consent from the patients, whole peripheral blood samples were collected for further genetic study.

2.2. DNA extraction

Genomic DNA from all patients was isolated from blood lymphocytes collected in EDTA vacutainers using standard salting out method. Three ml of blood was mixed thoroughly and incubated for erythrocyte lysis. After erythrocyte lysis, the packed white blood cells pellet was kept for Proteinase-k digestion overnight in the presence of 20% SDS. The genomic DNA was precipitated by the addition of 5 M NaCl and the DNA was spun down and washed with alcohol to remove salts and eluted with Tris-EDTA buffer [21]. DNA concentration and purity was assessed by optical density ratios (260/280 nm) using UV–vis Spectrophotometer (Lambda 35, Perkin Elmer, Waltham, Massachusetts, USA) and stored at −20 °C until use.

2.3. Sequence analysis

The referential genomic DNA sequence of *GATA4* was retrieved from Genbank (Accession No. NC_000008) and sequence specific primer pairs were designed to amplify the coding exons and exon-intron boundary regions of *GATA4* gene with the help of Primer Quest tool, IDT DNA software (https://www.idtdna.com/) and listed in Table 1. Polymerase chain reaction (PCR) was carried out with these specific primers using C1000 Touch Thermal cycler (Bio-Rad Laboratories, Hercules, California, USA). Reactions contained 100 ng of genomic DNA, 10× PCR buffer, 10 mM dNTPs, 10 picomole of each primer, 1.5 mM $MgCl_2$, and 1 unit *Taq* DNA polymerase (Bangalore Genei, India) and the conditions followed with an initial denaturation at 95 °C for 5 min followed by 33 cycles of denaturation at 95 °C for 30 s, annealing at 61 °C for 30 s, 72 °C for 30 s and final extension at 72 °C for 10 min. The PCR products were analyzed on 1% agarose gel and purified with GeneJET PCR Purification kit (Thermo Fisher Scientific). Sequence analysis was performed for all the six exons with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) under ABI-3500 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). DNA Sequences were analyzed by ABI Variant Reporter software version 1.1 (Applied Biosystems) using NG_008177 gene sequence as template. Variant sequences that were thus obtained were deposited in Clinvar.

2.4. Mutation prediction

Protein sequences of GATA4 protein from various species were

Table 3
Exonic variants in *GATA4* coding region.

Exon	Nucleotide change	Aminoacid change	dbSNP id	Mutation type	Mutation Prediction		
					Polyphen-2	SIFT	Mutation Taster
5	c.1064C > G	T355S	rs200167770	Missense	Benign	Tolerated	Polymorphism
5	c.1129A > G	S377G	rs3729856	Missense	Benign	Tolerated	Polymorphism
5	c.1138G > A	V380M	rs114868912	Missense	Benign	Tolerated	Polymorphism
6	c.1180C > A	P394T	rs200319078	Missense	Benign	Tolerated	Polymorphism
6	c.1273G > A	D425N	rs56208331	Missense	Probably damaging	Tolerated	Disease causing

Table 4A
Exonic and Intronic variants of *GATA4* gene in CHD patients.

	Gender	Sample Type	76885	83271	83502	85342	86268	87409	87813				
1	F	ASD	g.76885T > C	g.83271C > A									
2	F	ASD	g.76885T > C	g.83271C > A									
3	F	ASD, PFO	g.76885T > C	g.83271C > A									
4	M	ASD	g.76885T > C	g.83271C > A	g.83502A > M				g.87813A > W				
5	F	ASD	g.76885T > C	g.83271C > A	g.83502A > M				g.87813A > W				
6	M	ASD	g.76885T > C	g.83271C > A	g.83502A > M				g.87813A > W				
7	F	ASD	g.76885T > C	g.83271C > A	g.83502A > M				g.87813A > W				
8	F	ASD	g. 76885T > C	g.83271C > A	g.83502A > M				g.87813A > W				
9	F	ASD	g.76885T > C	g.83271C > A	g.83502A > M				g.87813A > W				
10	F	VSD	g.76885T > C	g.83271C > A	g.83502A > M				g.87813A > W				
11	M	VSD	g.76885T > C	g.83271C > A	g.83502A > M				g.87813A > W				
12	M	ASD, PDA	g.76885T > C	g.83271C > A	g.83502A > M				g.87813A > W				
13	F	ASD, PDA	g.76885T > C	g.83271C > A	g.83502A > M				g.87813A > W				
14	F	ASD, PDA	g.76885T > C	g.83271C > A	g.83502A > M				g.87813A > W				
15	F	PFO	g.76885T > C	g.83271C > A	g.83502A > M				g.87813A > W				
16	F	ASD,VSD	g.76885T > C	g.83271C > A	g.83502A > M				g.87813A > W	g.76937G > S			
17	M	ASD	g.76885T > C	g.83271C > A	g.83502A > C				g.87813A > W				
18	F	ASD	g.76885T > C	g.83271C > A	g.83502A > C				g.87813A > W				
19	M	ASD, PDA	g.76885T > C	g.83271C > A	g.83502A > C				g.87813A > W				
20	M	ASD,VSD	g.76885T > C	g.83271C > A	g.83502A > C				g.87813A > T				
21	M	VSD,TOF	g.76885T > C	g.83271C > A	g.83502A > C				g.87813A > T				
22	F	ASD	g.76885T > C	g.83271C > A	g.83502A > C				g.87813A > T				
23	NIL	ASD	g.76885T > C	g.83271C > A	g.83502A > C				g.87813A > T				
24	M	ASD,PDA	g.76885T > C	g.83271C > A					g.87813A > W	g.78343G > R			
25	F	ASD, VSD, PDA	g.76885T > C	g.83271C > A	g.83502A > M				g.87813A > W		g.85294C > Y		
26	M	ASD	g.76885T > C	g.83271C > A	g.83502A > M				g.87813A > W	p.T355S	g.84991G > R		
58	M	VSD	g.76885T > C	g.83271C > A					g.87813A > W		p.P394T	g.84991G > R	
59	F	VSD	g.76885T > C	g.83271C > A	g.83502A > M				g.87813A > W		p.P394T		
27	M	ASD, PDA	g.76885T > C	g.83271C > A	g.83502A > M				g.87813A > W	p.T355S			
28	F	ASD	g.76885T > C	g.83271C > A		g.85342C > Y	g.86268A > R	g.87409G > R					
29	F	VSD	g.76885T > C	g.83271C > A		g.85342C > Y	g.86268A > R	g.87409G > R					
30	F	PDA	g.76885T > C	g.83271C > A		g.85342C > Y	g.86268A > R	g.87409G > R					
31	M	ASD,PDA	g.76885T > C	g.83271C > A		g.85342C > Y	g.86268A > R	g.87409G > R					
32	NIL	ASD,VSD,PDA	g.76885T > C	g.83271C > A	g.83502A > M	g.85342C > Y	g.86268A > R	g.87409G > R					
33	F	ASD	g.76885T > C	g.83271C > A		g.85342C > Y	g.86268A > R	g.87409G > A					
34	F	ASD	g.76885T > C	g.83271C > A		g.85342C > Y	g.86268A > R	g.87409G > R			g.85294C > Y	**p.V380M**	
35	M	ASD	g.76885T > Y	g.83271C > M		g.85342C > Y	g.86268A > R	g.87409G > R		g.83415G > R	g.85294C > Y	**p.V380M**	
36	M	VSD	g.76885T > C	g.83271C > A		g.85342C > T		g.87409G > A					
37	M	ASD	g.76885T > C	g.83271C > A		g.85342C > T		g.87409G > A					
38	M	ASD,PDA	g.76885T > C	g.83271C > A			g.86268A > R	g.87409G > R			p. P394T		
39	NIL	ASD, VSD	g.76885T > C			g.85342C > Y	g.86268A > G	g.87409G > R	g.87813A > W	g.76937G > S	p.S377G		
40	M	ASD,VSD	g.76885T > C	g.83271C > A	g.83502A > M	g.85342C > Y	g.86268A > R	g.87409G > R	g.87813A > W				
41	M	PDA	g.76885T > C	g.83271C > A	g.83502A > M	g.85342C > Y	g.86268A > R	g.87409G > R	g.87813A > W				
42	M	PDA	g.76885T > C	g.83271C > A	g.83502A > M	g.85342C > Y	g.86268A > R	g.87409G > R	g.87813A > W				
43	M	ASD	g.76885T > C	g.83271C > A	g.83502A > M	g.85342C > Y	g.86268A > R	g.87409G > R	g.87813A > W				
44	F	ASD	g.76885T > C	g.83271C > A	g.83502A > M	g.85342C > Y	g.86268A > R	g.87409G > R	g.87813A > W		g.83318G > K		
45	F	VSD	g.76885T > Y	g.83271C > M	g.83502A > M	g.85342C > Y	g.86268A > R	g.87409G > R	g.87813A > W		g.83415G > R		
46	F	VSD	g.76885T > Y	g.83271C > M	g.83502A > M	g.85342C > Y	g.86268A > R	g.87409G > R	g.87813A > T		g.83415G > R		
47	F	VSD	g.76885T > C	g.83271C > A	g.83502A > M	g.85342C > Y	g.86268A > R	g.87409G > R	g.87813A > W				
48	M	PS	g.76885T > C	g.83271C > A	g.83502A > M	g.85342C > Y	g.86268A > R	g.87409G > R	g.87813A > W				
49	M	ASD,PDA	g.76885T > Y	g.83271C > M		g.85342C > Y	g.86268A > R	g.87409G > R	g.87813A > T				
50	M	ASD, VSD, PDA	g.76885T > C	g.83271C > A	g.83502A > M	g.85342C > Y	g.86268A > R	g.87409G > R	g.87813A > W		g.87725T > Y		
51	M	VSD, DORV	g.76885T > C	g.83271C > M			g.86268A > R		g.87813A > W	g.78343G > R	g.76937G > S	**g.83073T > Y**	
52	F	TOF, VSD, PS	g.76885T > C	g.83271C > M	g.83502A > M		g.86268A > R		g.87813A > T	g.76937G > S	p.S377G		

(continued on next page)

Table 4A *(continued)*

	Gender	Sample Type	76885	83271	83502	85342	86268	87409	87813				
53	F	ASD,VSD,PDA	g.76885T > Y		g.83502A > M		g.86268A > R		g.87813A > W				p. D425N
54	M	VSD	g.76885T > C	g.83271C > M	g.83502A > M		g.86268A > R		g.87813A > T	g.76937G > S			p.S377G
56	F	ASD	g.76885T > C	g.83271C > M	g.83502A > M		g.86268A > R		g.87813A > W	g.76937G > S			p.D425N
55	M	ASD	g.76885T > C	g.83271C > M			g.86268A > G		g.87813A > T	g.76937G > C			p.S377G
57	F	VSD	g.76885T > C	g.83271C > A		g.85342C > Y		g.87409G > R	g.87813A > W				p.P394T
60	F	VSD	g.76885T > C	g.83271C > M	g.83502A > M		g.86268A > R			g.76937G > S			p.S377G

obtained from NCBI protein database and aligned using MUSCLE program (www.ebi.ac.uk/Tools/msa/muscle/). PolyPhen-2 (Polymorphism Phenotyping, http://genetics.bwh.harvard.edu/pph2/), SIFT (Sorting Intolerant From Tolerant, http://sift.jcvi.org/) and MutationTaster (www.mutationtaster.org) programs were utilized to validate the mutation prediction of variants.

2.5. In silico analysis

The intronic mutations, RNA splice sites and RNA branch points of *GATA4* gene were analyzed by using Human Splicing Finder (HSF) [22] software version 3.0 (www.umd.be/HSF3/), an online bioinformatics tool to predict the effects of mutations on splicing signals or splicing motifs either to enhance or suppress splicing such as Exonic Splicing Enhancers (ESEs), Exonic Splicing Silencers (ESSs), Intronic Splicing Enhancers (ISEs) and Intronic Splicing Silencers (ISSs).

3. Results

The patient population with CHD in this study included 22 ASD, 12 VSD, 9 ASD and PDA, 4 ASD and VSD, 4 ASD, VSD and PDA, 3 PDA, one each with PS, PFO, ASD with PFO, VSD with Double Outlet Right Ventricle (DORV), VSD with TOF as well as VSD, TOF with PS (Table 2). 10 samples without CHD were included as controls in the study.

Mutational analysis of CHD cases revealed twenty variants including five in the coding and fifteen in the non-coding region (Tables 3, 4A, and 4B). The data analysis of controls also presented intronic variants however the genotypic pattern were different from the cases under study group (Table 5).

There were five known heterozygous missense mutations (p.T355S, p.S377G, p.V380M, p.P394T and p.D425N) identified in exon 5 and exon 6 regions (Fig. 1A). Multiple sequence alignment of *GATA4* sequence from various species was aligned and only p.D425N showed the evolutionarily conserved throughout the species. Mutation prediction results showed that the p.T355S, p.S377G, p.V380 M and p.P394T variants were predicted to be benign, tolerated and polymorphism by PolyPhen-2, SIFT and MutationTaster. Only p.D425N variant was predicted to be probably damaging, tolerated and disease causing (Table 3).

Apart from these missense variants, there were 15 novel intronic variants (Fig. 1B) found in different CHD phenotypes as listed in Tables 4A and 4B. These intronic variants were analyzed for potential branch point and potential ISEs and ISSs. The results indicated two variants predicted which could be potential branch point sites. One potential branch point site at g.83271C > A/M variant with GCCTAAG motif of 83.82 consensus value in wild type and in addition, this mutation leads to formation of ISS motif 2 (AGAGTGCA) with 63.85 consensus value (Fig. 2A) and another potential branch point site at g.86268A > R variant with GGCTGAA motif of 82.24 consensus value in wild type. These intronic mutations at the potential branch point are predicted to be disrupted (Fig. 2B).

The g.76885T > C/Y intronic variant leads to formation of new ISE motif for SRp40; whereas the g.76937G > S and g.83073T > C/Y variants lead to the disruption of SRp40 and SRp55 ISE motifs respectively. On the other hand, g.78343G > A/R variant leads to the disruption of ISS motif 1 (CAAAACGT) and g.87813A > T/W leads to formation of ISS motif 2.

The g.83318G > K variant leads to the formation of SF2/ASF ISE motif and ISS Motif 2, whereas g.85294C > Y variant leads to the disruption of SRp40 ISE motif and formation of ISS motif 2. The g.84991G > R variant leads to the disruption of ISS motif 1 and formation of ISS motif 2 and the g.87725T > Y variant leads to the

Table 4B
Exonic and Intronic variants of *GATA4* gene in control group.

	Gender	Sample Type	76885	83271	83502	85342	86268	87409	87813	
1	F	Control1	g.76885T > C	g.83271C > A						
2	M	Control2	g.76885T > C	g.83271C > A	g.83502A > C					
3	F	Control3	g.76885T > C	g.83271C > A	g.83502A > M					
4	Nil	Control4	g.76885T > C		g.76937G > S		g.86268A > R			p.S377G
5	F	Control5	g.76885T > C	g.83271C > A		g.85342C > T	g.86268A > G			
6	M	Control6	g.76885T > C	g.83271C > A	g.83502A > M		g.86268A > R	g.87409G > R	g.87813A > T	
7	M	Control7	g.76885T > C	g.83271C > A			g.86268A > R	g.87409G > R		
8	M	Control8	g.76885T > C	g.83271C > A	g.83502A > M	g.85342C > Y	g.86268A > R	g.87409G > R	g.87813A > W	
9	F	Control9	g.76885T > C	g.83271C > A	g.83502A > M	g.85342C > Y	g.86268A > R	g.87409G > R	g.87813A > T	g.78042G > R
10	Nil	Control10	g.76885T > Y	g.83271C > M	g.83502A > M	g.85342C > Y	g.86268A > R	g.87409G > R	g.87813A > W	g.83415G > R

Table 5
Genotypic pattern of *GATA4* gene at 85342, 86268, 87409 and 87813 positions in CHD patients (5A) and control group (5B).

A: CHD patients

S.No	Frequency	85342	86268	87409	87813
1.[a]	3	CC	AA	GG	AA
2.	22	CC	AA	GG	AT
3.	4	CC	AA	GG	TT
4.	7	CT	AG	AG	AA
5.	1	CT	AG	AA	AA
6.	2	TT	AA	AA	AA
7.	1	CC	AG	AG	AA
8.[a]	11	CT	AG	AG	AT
9.	1	CT	GG	AG	AT
10.	3	CC	AG	GG	AT
11.	2	CC	AG	GG	TT
12.	1	CC	GG	GG	TT
13.	1	CT	AA	AG	AT
14.	1	CC	AG	GG	AA

B: Control group

S.No	Frequency	85342	86268	87409	87813
1.[a]	3	CC	AA	GG	AA
2.	1	CC	AG	GG	AA
3.	1	TT	GG	GG	AA
4.	1	CC	AG	AG	TT
5.	1	CC	AG	AG	AA
6.[a]	2	CT	AG	AG	AT
7.	1	CT	AG	AG	TT

[a] Genotypic patterns are identical in both CHD patients and control group.

formation of SF2/ASF ISE motif and ISS motif 1.

The g.83415G > R variant leads to formation of SRp40 ISE motif and formation of ISS motif 3 and disruption of SF2/ASF2 ISE motif; g.85342C > T/Y leads to formation of SRp55 ISE motif and formation of ISS motif 2. The g.87409G > A/R leads to formation of SRp40 ISE motif and disruption of ISS motif 1. However, intronic variant g.83502A > C/M revealed no significance to the formation of potential branch point site or change of ISE and ISS (Table 6).

4. Discussion

Intronic variants and its consequence on the splicing mechanism can be better understood by using *in silico* analysis that includes different algorithms to identify various regulatory elements. In the present study, HSF3.0 *in silico* analysis showed that the intronic variant g.86268A > R predicted as potential branch point site. Consensus wild type score for the potential branch point ranges from 0 to 100 and the threshold value is defined to be 67. Scores above 67 is considered to be potential branch point. When a mutation occurs, if the wild type score is above 67 and the score variation (between wild type and mutant) decreases by more than 10%, it is considered that the mutation breaks the branch point. In the intronic variant, g.83271C > A/M apart from the loss of potential branch point, it also lead to the formation of ISS motif 2. Alterations in the branch point position might provoke serious defects in pre-mRNA splicing leading to exon skipping or intron inclusion or activation of cryptic splice sites [23–25].

Pre-mRNA splicing is an essential process in gene expression and majority of human genes undergo alternative splicing, leading to the formation of isoforms [26]. It is regulated by *cis*-elements (ESE, ESS, ISE and ISS) and *trans*-acting factors such as Serine and Arginine-rich proteins (SR proteins) and heterogeneous nuclear ribonucleoprotein (hnRNP) [27]. ESEs/ESSs will promote or inhibit inclusion of exon,

Fig. 1. (A) Sequence chromatogram indicating five heterozygous missense mutation (p.T355S, p.S377G, p.V380M, p.P394T and p.D425N). (B) Sequence chromatogram showing 15 novel intronic variants.

where as ISEs/ISSs will enhance or inhibit adjacent splice sites usage [27]. ESEs/ISEs are characterized as small RNA motifs that act as binding sites for SR proteins to enhance splicing by spliceosome recruitment [28]. ESSs/ISSs will bind to negative regulators including hnRNP family to oppose the positive effect of SR proteins and repress splicing [29–31].

SR proteins are RNA-binding proteins consists of C-terminal domain with highly conserved amino acid sequences (RS domain) and N-terminal RNA recognition domain (RRM domain) [32]. Presently, nine human SR proteins have been identified [33] and its characteristic feature is the capability to regulate 5′ splice site choice [32]. Among SR proteins, SF2/ASF, SRp40 and SRp55 were well-studied in alternative splicing. SR proteins act as splicing activators and can cooperate and compete together in splicing regulation [33].

SF2/ASF (SRSF1) was the first identified and most abundant protein which transfers between the nucleus and cytoplasm [34] and its consensus sequence is SRSASGA (S indicates G/C, R indicates Purine), rich with purine residues [33]. SRp40 (SRSF5) and SRp55 (SRSF6) are characterized with C-terminal (RS domain), RRM domain and RRM homology domain [35]. SRp40 yields the consensus sequence ACDGS (D indicates other than C, S indicates G/C) and SRp55 has consensus sequences USCGKM (S indicates G/C, K indicates U/G, M indicates A/C) [33,35]. SRp40 ESEs/ISEs are also activated by SRp55 because they are closely related in domain structures and molecular mass of 31.2 kD, 39.6 kD and 65% sequence identity [33]. At higher concentrations of SF2/ASF, it will help to use the sites which are proximal to 3′ splice site, whereas SRp40 and SRp55 will stimulate to use of distal sites and further expands the association of U1 snRNP with distal sites [32].

ISEs/ISSs are with primary importance in splicing regulation, due to the conserved sequences surrounding constitutive exons, around a

Heptamer position	Heptamer cDNA position	Branch Point position	Branch Point cDNA position	Branch Point motif	Consensus value (0-100)
44	+44	49	+49	CCCTCAG	97.27
46	+46	51	+51	CTCAGAG	73.89
54	+54	59	+59	GCCTAAG	83.82
117	+117	122	+122	ATC9GAC	72.36
133	+133	138	+138	CTTTCAG	70.07
152	+152	157	+157	AGCCCAG	79.41
206	+206	211	+211	GGCTCAA	87.17
211	+211	216	+216	AACTCAA	82.27
224	+224	229	+229	GCCTGAT	89.54
234	+234	239	+239	TGCCGAC	79.93
247	+247	252	+252	GACCCAG	79.74
254	+254	259	+259	TGCTCAG	91.17
269	+269	274	+274	AGCCGAG	74.48

A

Heptamer position	Heptamer cDNA position	Branch Point position	Branch Point cDNA position	Branch Point motif	Consensus value (0-100)
1002	+1002	1007	+1007	TGCTCAG	91.17
1013	+1013	1018	+1018	AGCTGAT	82.34
1020	+1020	1025	+1025	GCATCAC	72.65
1024	+1024	1029	+1029	CACCCAG	81.37
1026	+1026	1031	+1031	CCCAGAC	79.98
1033	+1033	1038	+1038	CCTTCAT	72.27
1039	+1039	1044	+1044	TGCCTAG	70.31
1091	+1091	1096	+1096	TCCTGAG	90.81
1098	+1098	1103	+1103	GGCTGAA	82.24
1136	+1136	1141	+1141	CCCTCCC	70.37
1139	+1139	1144	+1144	TCCCCAG	86.72
1153	+1153	1158	+1158	CTCCCAA	80.98
1154	+1154	1159	+1159	TCCCAAG	74.89
1161	+1161	1166	+1166	CCCTCAG	97.27
1182	+1182	1187	+1187	GCCTCAT	94.48

B

Fig. 2. *In silico* analysis of intronic variants by HSF3.0. (A) Potential branch point showing consensus value of g.83271C > A/M variant (B) Potential branch point showing consensus value of g.86268A > R variant.

distance of 150 bp or more in mammals [36]. Among ISSs, motif 2–(U/G)G(U/A)GGGG proved as a strong splicing silencer than motif 1(CTAGAGGT) and motif 3 (TCTCCCAA), where it predicted to wield too weak silencing effects for making any variation in splicing [28]. Another two groups identified Fox-1/2 binding motif (UGCAUG) with more conserved sequence, associated with tissue-specific splicing factors [37,38].

In the present study, the variants g.85342C > T/Y, g.86268A > G/R, g.87409G > A/R and g.87813A > T/W were observed in both cases and controls. Interestingly, these genotypic patterns observed in cases were not identical with the controls except two patterns as represented in Tables 4A and 4B. This observation suggested the possibility of other intronic variants presented responsible for the development of CHD. The other variants namely g.76937G > S, g.78343G > A/R and g.83073T > C/Y were seen in VSD with DORV condition and in PDA, apart from g.76885T > C/Y, g.83271C > A/M, g.86268A > R, and g.87813A > T/W variants, the g.87409G > A/R was picked up consistently in all cases. In one VSD, there were two missense heterozygous mutations (p.T355S and p.S377G) along with other intronic variants.

To the best of our knowledge, all these potential branch point mutations, mutations in ISEs and ISSs have not been presented in the literature. In the present study, we could not establish the role of these intronic variants to different clinical condition of CHDs and thus it could be attributed essentially to mutations in other transcription factors. These mutational studies in other genes or whole genome sequencing in CHD patients might shed light on the clinical spectrum of the disease.

5. Conclusion

In conclusion, this study expands the spectrum of *GATA4* intronic mutations in Indian population and provides additional information on molecular aberration seen in CHD patients.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Table 6
Branch point analysis showing Potential branch points, Enhancer motifs (ISE) and Silencer motifs (ISS) for fifteen intronic variants.

Intron	Intronic mutations	Mutation frequency in subjects	Branch Point Analysis					
			Potential branch point		Enhancer motifs		Silencer motifs	
			Wild type	Mutant type	Wild type	Mutant type	Wild type	Mutant type
1	g.76885T > C	55/60	–	–	–	Formation of SRp40 binding motif 81.26 (78.08)	–	–
	g.76885T > Y	5/60						
1	g.76937G > C	2/60	–	–	SRp40 binding motif 79.70 (78.08)	Disruption of SRp40 binding motif	–	–
	g.76937G > S	7/60						
3	g.78343G > R	2/60	–	–	–	–	Motif 1 61.17 (60)	Disruption of Motif 1
3	g.83073T > Y	1/60	–	–	SRp55 binding motif 77.0(73.86)	Disruption of SRp55 binding motif	–	–
4	g.83271C > A	48/60	GCCTAAG 83.82 (67)	Disruption of GCCTAAG site	–	–	–	Formation of Motif 2 63.85 (60)
	g.83271C > M	10/60						
4	g.83318G > K	1/60	–	–	–	Formation of SF2/ASF binding motif 77.15 (72.98)	–	Formation of Motif 2 70.71 (60)
4	g.83415G > R	3/60	–	–	SF2/ASF2 binding motif 72.15 (72.98)	Disruption of SF2/ASF2 & Formation of SRp40 binding motifs 85.51 (78.08)	–	Formation of Motif 3 63.50 (60)
4	g.83502A > C	7/60	No significant mutation prediction					
	g.83502A > M	33/60						
4	g.84991G > R	2/60	–	–	–	–	Motif 1 70.15 (60)	Disruption of Motif 1 & Formation of Motif 2 66.40 (60)
5	g.85294C > Y	3/60	–	–	SRp40 binding motif 84.97(78.08)	Disruption of SRp40 binding motif	–	Formation of Motif 2 62.75 (60)
5	g.85342C > T	2/60	GCCGCAC 83.28 (67)	GCCGCAC −79.39	–	Formation of SRp55 binding motif 77.19 (73.86)	–	Formation of Motif 2 68.15 (60)
	g.85342C > Y	21/60						
5	g.86268A > G	2/60	GGCTGAA 82.24 (67)	Disruption of GGCTGAA site	–	–	–	–
	g.86268A > R	26/60						
6	g.87409G > A	3/60	–	–	–	Formation of SRp40 binding motif 78.98 (78.08)	Motif 1 65.06 (60)	Disruption of Motif 1
	g.87409G > R	21/60						
6	g.87725T > Y	1/60	–	–	–	Formation of SF2/ASF binding motif 92.18 (72.98)	–	Formation of Motif 1 72.64 (60)
6	g.87813A > T	9/60	–	–	–	–	–	Formation of Motif 2 61.41 (60)
	g.87813A > W	36/60						

*Threshold value of ISEs and ISSs motif values are given in parentheses.

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Case Report

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Molecular Delineation of Partial Trisomy 14q and Partial Trisomy 12p in a Patient with Dysmorphic Features, Heart Defect and Developmental Delay

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Key Words
Array CGH · Balanced translocation · Partial trisomy 12p · Partial trisomy 14q

Abstract
This study describes a molecular analysis of partial trisomy 14q and partial trisomy 12p in a 5-year-old male child presenting with dysmorphic features, congenital heart disease and global developmental delay. Chromosomal analysis of the patient with GTG bands revealed a 47,XY,+der(14)t(12;14)(p13;q22)mat karyotype; the mother's karyotype was 46,XX,t(12;14)(p13;q22). Further, oligonucleotide array-CGH studies revealed an amplification of 32.3 Mb in the 14q11.1q22.1 region, substantiating partial trisomy 14q and additionally displaying an amplification of ~1 Mb in the 12p13.3pter region for partial trisomy 12p. This is the first study to demonstrate a novel association of partial trisomies of 14q and 12p due to a 3:1 segregation of a maternal balanced translocation involving chromosomes 12 and 14. Gene ontology studies indicated 5 potential candidate genes in the amplified regions for the observed congenital anomalies.

Congenital malformations due to various types of chromosomal aberrations have been described extensively in the literature [Schinzel, 2001]. Complete trisomy of chromosome 14, a rare chromosomal disorder, is frequently associated with spontaneous abortions, and on the other hand, in the presence of a normal cell line, it appears to be compatible with live birth [Kajii et al., 1972]. The life expectancy of patients with partial trisomy 14 depends upon the chromosomal segment involved in the structural abnormality [Smith et al., 1980; Dundar et al., 2011]. Similarly, trisomy 12p is also a rare chromosomal disorder with the incidence of 1:50,000 births. Little is known in the literature about the clinical features or the life expectancy of children affected by trisomy 12p aberrations [Stengel-Rutkowski et al., 1981; Segel et al., 2006]. Generally, these types of partial chromosomal aberrations present with multiple congenital and dysmorphic anomalies besides developmental delay with or without mental retardation [Liu et al., 2012; Salas-Labadía et al., 2014].

Traditional methods of chromosome analysis can identify abnormalities up to a limit of 5 Mb and have a limited scope to identify submicroscopic deletions and amplifications, particularly in nonsyndromic malforma-

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tions. Array CGH as a tool for molecular genetic analysis to understand the basis of congenital malformations is a rapidly developing trend, as evidenced by a large number of reports from several countries [Aradhya et al., 2007; Baris et al., 2007; Almal and Padh, 2012]. Here, we report a case of a 5-year-old male child with partial trisomy 14q, as shown by conventional karyotyping and array-CGH studies, confirming the results and further revealing an additional partial trisomy 12p.

Case Report

Patient Details

A 5-year-old male child was presented with fever, cough and dysmorphic features. He is the second child born to a nonconsanguineous couple. His medical records indicated the following: birth weight ~2.5 kg, the presence of meconium-stained liquor and that he cried after resuscitation. At the age of 4 years, he was diagnosed with atrial septal defect and pulmonary stenosis with a history of respiratory as well as feeding difficulties with global developmental delay.

Physical examination revealed that his head circumference was 46 cm (<5th centile), weight 13 kg (<5th centile) and his height 98 cm (<5th centile). Clinical examination revealed dysmorphic features such as microcephaly, low-set ears with prominent pinnae, a prominent nose with a broad nasal bridge, and a high-arched palate besides retrognathia with mental retardation. The child also had tachycardia, tachypnea and nutritional anemia. Neurological examination revealed hypertonia and increased deep tendon reflexes. Unfortunately, the child passed away 2 days after hospital admission due to aspiration pneumonia. No further clinical information was available regarding this patient.

Cytogenetic and Molecular Analysis

A peripheral blood sample was taken from the patient and referred to our laboratory for chromosomal analysis. Metaphase chromosomes were obtained from a PHA-stimulated lymphocyte culture by standard technique [Seabright, 1971]. Chromosomal analysis was carried out by GTG banding at 400–450 band-level resolution in the patient and parents. The G-banded chromosomes were analyzed and interpreted according to the International System for Human Cytogenetic Nomenclature 2013 [ISCN, 2013]. Further, array CGH was also performed.

Genomic DNA was extracted from peripheral blood using the standard salting-out procedure [Miller et al., 1988]. The DNA concentration was determined using Nanodrop spectrophotometer (ND-1000, Thermo Fisher Scientific, Waltham, Mass., USA). Array CGH was performed by using Agilent Human 1×1M Oligonucleotide CGH array (AMAMDID 21529, Agilent Technologies Inc., Santa Clara, Calif., USA) containing about 962,029 probes with an overall median probe spacing of 2.1 kb. Analysis was performed in accordance with the manufacturer's protocol. The microarray slide was scanned using Agilent scanner (design G4900DA, Agilent Technologies), and the data were processed and analyzed by means of Agilent CytoGenomics 2.7 version software with the statistical algorithm ADM-1, with the threshold of 6.0 and a 2-probe minimum aberration call with an average log ratio of 0.25 to identify chromosome aberrations.

Results

GTG banding revealed 47 chromosomes with a marker chromosome in all the analyzed metaphases of the proband. This marker chromosome appeared to be acrocentric, which was smaller than the D-group and larger than the G-group chromosomes. QFQ banding was performed to identify the marker chromosome, and it demonstrated the presence of a single normal Y chromosome. Parental chromosomal analyses revealed that the mother was a carrier of a balanced translocation 46,XX,t(12;14)(p13;q22) and the father had a normal 46,XY karyotype. The analyses also confirmed the maternal origin of marker chromosome (14q–) in the patient. The child's karyotype was 47,XY,+der(14)t(12;14)(p13;q22)mat and the mother's karyotype was 46,XX,t(12;14)(p13;q22) (fig. 1).

In our patient, array-CGH analysis confirmed the partial trisomy 14q with the amplification of 32,313,772 bases (18,505,611–50,819,383) in the q11.1q22.1 region of chromosome 14 represented by 10,312 probes on the arrays. Chromosome 12 also showed amplification of 1,000,846 bases (59,822–1,060,668) in the terminal region (12p13.3pter) represented by 436 probes, suggesting partial trisomy of 12p (fig. 2). This partial trisomy 12p segment was undetectable by conventional karyotyping as the amplified segment was ~1 Mb. Array CGH of the mother revealed that there was no major loss or gain of genetic material indicating that the net genomic content has remained intact in the mother, which supports the clinically normal phenotype of mother, despite being a carrier of balanced translocation.

Discussion

The advent of the chromosomal microarray technique has facilitated the identification of an increasing number of submicroscopic chromosomal deletions and duplications which have been associated with a variety of congenital anomalies. To the best of our knowledge, this is the first study to report the combined presence of partial trisomy 14q and partial trisomy 12p in a child consequential to maternal balanced translocations involving chromosomes 12 and 14 by employing the array-CGH technology. The molecular mechanism of the formation of partial trisomy14q is suggested to be due to suppression of chiasma formation at the centromere region; the unstable bivalent formed between the normal and the abnormal chromosome 14 predisposes to premature desynapsis or asynapsis and the subsequent segregation of the 2 chromosomes 14

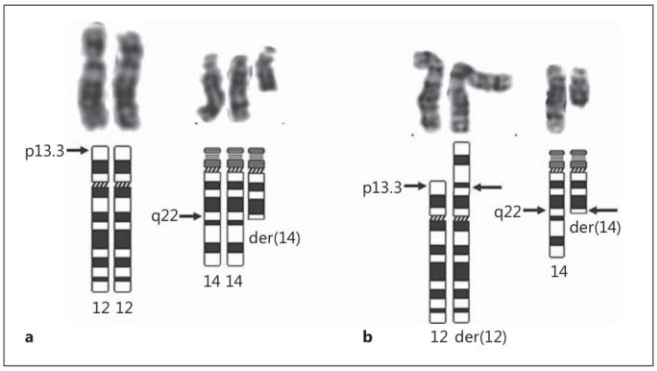

Fig. 1. a GTG banding showing partial trisomy 14q, 46,XY,+der(14)t(12; 14)(p13;q22)mat, of the patient. Arrows indicate translocation breakpoints. **b** The mother's partial karyotype showing a balanced translocation 46,XX,t(12; 14)(p13;q22). Arrows indicate translocation breakpoints and break-reunion junction.

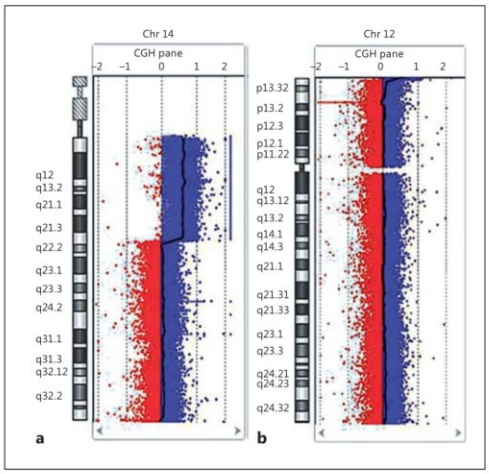

Fig. 2. Array-CGH profile revealing partial trisomy 14q with amplification in the 14q11.1q22.1 region (**a**) and partial trisomy 12p with amplification in the 12p13.3pter region (**b**).

Table 1. Clinical manifestations in the present case correlated with common features in both partial trisomy 14q and partial trisomy 12p

Clinical features	Partial trisomy		Present case
	12p[a]	14q[b]	
Birth weight	normal/high	low	normal
Developmental delay	+	+	+
Microcephaly	–	+	+
Macrocephaly	+	–	–
Low-set/malformed ears	+	+	+
Prominent nose or broad nasal bridge	+	+	+
Micrognathia/retrognathia	–	+	+
Cardiac defect	+	+	+
Mental retardation	+	+	+

[a] Allen et al., 1996; Rauch et al., 1996; Chen et al., 1997; Tekin et al., 2001; Shashidhar Pai et al., 2003; Tsai et al., 2005, Liu et al., 2012.

[b] Allderdice et al., 1971; Fryns et al., 1974; Cohen et al., 1975; Simpson and Zellweger, 1977; Smith et al., 1980; Angle et al., 1999; Shashidhar Pai et al., 2003.

to the same spindle [Cohen et al., 1975]. The probability of a 14q proximal partial trisomy zygote has been shown to be higher in female balanced translocation carriers than in male carriers [Valkova and Stefanova, 1993]. The results of the present study also indicate that the origin of the derivative chromosome is due to a 3:1 segregation during meiosis resulting in an unbalanced aneuploidy [Angle et al., 1999].

Comparing cases of partial trisomy 14q and 12p previously reported in literature with the present case revealed a combination of the clinical features found in our patient which are depicted in table 1.

The array-CGH study showed an amplification of ~32.3 Mb in the 14q region and an amplification of somewhat more than 1 Mb in the 12p region, confirming partial trisomy 14q and partial trisomy 12p. These amplified

regions of the patient comprise of 237 genes on chromosome 14 and 11 genes on chromosome 12. Gene ontology studies indicate that several of these genes are involved in multiple signaling pathways (online suppl. table 1, see www.karger.com/doi/10.1159/000381294).

To establish the genotype-phenotype correlations, an extensive search in the OMIM database was performed for the amplified genes in the 14q region. This led us to consider the *MYH6* (OMIM 614089), *FOXG1* (OMIM 164874), *SUPT16H* (OMIM 613457), and *MGAT2* (OMIM 602616) genes, which are closely associated with abnormal phenotypes such as dysmorphic features, mental retardation and developmental delay.

MYH6, a cardiac muscle-specific myosin gene has been shown to be closely associated with atrial septal defect *(ASD3)* and familial hypertrophic cardiomyopathy. Unregulated expression of *MYH6* due to *TBX5* (a cardiac transcription factor) mutation leads to Holt-Oram syndrome [Ching et al., 2005].

FOXG1 is a transcription factor closely associated with Rett syndrome, which regulates the development of the telencephalon from early embryonic to adult stages by multiple and diverse mechanisms. It has also been shown to have a role in brain development and has been recognized as a strong candidate gene to explain the developmental disorder associated with deletion in the 14q12 region [Kortüm et al., 2011].

SUPT16H encodes the large subunit of the conserved FACT complex. Haploinsufficiency of S*UPT16H* has been associated with congenital anomalies, developmental delay and mental retardation [Chelly et al., 2006; Zahir et al., 2007]. The *MGAT2* gene encodes UDP-N-acetyl glucosamine, a Golgi enzyme that catalyzes the conversion of oligomannose to complex N-glycans. Homozygous Lys237-to-Asn (K237N) mutation in the *MGAT2* gene has been associated with distinct dysmorphic features such as microcephaly, retrognathia, prominent columella, prominent nasal bridge, thin upper lip, diastema, and mental retardation [Alazami et al., 2012].

The amplified region of 12pter lies close to the 12p13.3p13.1 region, which has been implicated in 12p13.33 microdeletion syndrome and could be critical for the facial features [Rauch et al., 1996]. Among the 11 genes from this region, the *ERC1* gene encodes a protein that belongs to the family of RIM-binding proteins involved in the regulation of neurotransmitter release. *ERC1* has also been recognized to be responsible for speech sound disorder, childhood apraxia of speech and developmental verbal dyspraxia [Thevenon et al., 2013]. Further, *ERC1* has been found fused to the *RET* gene by rearrangement due to the translocation t(10;12)(q11;p13) in thyroid papillary carcinoma [Nakata et al., 2002].

Inherent duplications of some regions of the chromosomes involving strong candidate genes could present with multiple congenital anomalies [Patnala et al., 2013]. However, these conditions have been neither described succinctly nor compared with deletions in equivalent regions of the same chromosomes to decipher the roles of the deletion or amplification to the observed phenotypes. The phenotypic presentations in our patient could be attributed to the amplified genes but with the support of additional molecular events taking place in specific tissues or organs. Nonetheless, the emphasis on the candidate genes in the amplified region could not be ignored because of the functions detailed in the literature as discussed above.

Employing the array-CGH technique, we were able to establish the trisomies of 14q and 12p and to correlate the molecular underpinnings of the abnormal clinical presentations of the patient, either to the trisomies or to the genes located in the amplified regions. However, further epigenetic and gene expression studies need to be done to yield more definitive explanations to the etiopathogenesis of the congenital anomalies observed in our patient. The scope of such studies is limited at this stage; therefore, it is difficult to confirm or negate the roles the candidate genes play in causing the abnormal phenotypic features observed in our patient, who passed away shortly after hospital admission. Furthermore, this study highlights the relevance of array CGH in providing clinical and diagnostic results and cues for proper genetic counseling. The results of this study illustrate the possibility of having missed many submicroscopic chromosomal aberrations in several of the congenital anomalies already reported in the literature.

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CHAPTER-8

Summary and Conclusion

This study considered the spectrum of congenital anomalies observed in this region which seemed to be more unusually frequent than expected which probably could be attributed to various factors such as cultural, environmental, occupational and nutritional status. The observations of this study were encouraging for creation of a birth registry of dysmorphia with demographic details which might enable to correlate the findings with the probable factors responsible for the congenital anomalies observed in this region. Though conventional cytogenetic studies were able to identify a few abnormal chromosomal patterns in cases presented with global developmental delay, however it failed to provide a cue on chromosomal abnormalities at least in some cases where phenotypic presentations were suggestive of genetic causes. Array CGH studies conducted led to identification of deletions or duplications and further analysis of these regions indicated the candidate genes that were missing or duplicated. This approach enabled the identification of some unique patterns in the chromosomes responsible for Partial Trisomy 14q and 12p as well as 832 kb deletion in *VPS13B* gene of Cohen syndrome.

Studies on CHD revealed the importance of intron-exon boundary regions of the *GATA4* gene mutations which provide additional information on molecular aberration seen in CHD patients. It is a noteworthy finding in this study. On the contrary, the sequence variant search in *NKX2-5* gene did not yield any interesting finding limiting the scope of mutational analysis of the gene. This study suggests that *NKX2-5* gene mutations could be conserved and specific. However, studies through targeted panel sequencing or next generation sequencing in a larger population might shed light to understand the multifactorial etiology of CHD.

The oxidative DNA damage in CHDs, revealed the increased levels of 8-OHdG in combination of heart defects comprising ASD, VSD and PDA. The elevated levels of 8-OHdG in

the combination cases PDA with septal defects and a comparatively shorter life span in individuals suffering from this defect could be attributed to possible transversion mutations leading to disturbances in the translation process resulting in fatal clinical manifestations.