A Study of Birth Defects: Genetic and Epigenetic Determinants

Chapter 5: Summary and Conclusion
Congenital malformation or birth defect is a serious health issue of present time that has troubled the globe with a great health concern. As defined by WHO, birth defects are structural or functional (e.g. metabolic disorders) anomalies that originate in the fetus during intrauterine life and can be identified prenatally. According to joint World Health Organization (WHO) and March of Dimes (MOD) meeting report on birth defects, the problem is more prevalent in the middle and low-income countries with an annual incidence of approximately 7.9 million. In India, approximately 61 to 69.9 in 1000 live births are detected with the genetic anomaly. Among various types of birth defects, Down syndrome (DS) is characterized by intellectual disability and several other phenotypic and clinical anomalies. Globally, DS alone accounts for 15-20% of intellectual disability.

Another important congenital malformation is congenital heart disease (CHD). It is defined as any anatomical malfunction of heart or under development of blood vessels near the heart at the time of birth. The frequency of occurrence of congenital cardiac defects varies between 0.47 to 1.17% of live births. It is believed that about 80% of CHD is multifaceted and arises due to diverse combinations of genetic and environmental factors. Ventricular Septal Defect (VSD) and tetralogy Of Fallot (TOF) both are frequent type of CHD in newborn. Cardiac development involves a controlled pathway comprising of various developmental pathway molecules, transcription factors, and epigenetic regulators. Mutations in these genes may lead to disruption of normal signaling cascades leading to CHD. The reported risk factors for CHD include various factors such as the use of certain drugs, such as thalidomide, retinoic acid, and exposure to organic solvents. A strong association between DS and CHD has been well reported. The prevalence of CHD is 40-50 times higher in children with DS.

It has been shown that both the conditions have many common etiologies and risk factors. The knowledge of these causes and risk factors can provide useful information to modify, manage or even prevent the conditions. Therefore, our study aimed at redefining the etiology and risk factors for a birth defect, DS and CHD. We have investigated the possible risk factors, etiologies and epigenetic influence.
Part-A: Down syndrome in young Indian parents: A study of possible risk factors

The karyotyping analysis results were in accordance with the previous study with 208 postnatal karyotyping studies of DS. The risk of occurrence of DS was found to be independent of parental age, however, the occupation and environmental exposure might have played a pivotal role in the risk of occurrence of meiotic non-disjunction. Earlier studies have presented some proof supporting the relationship between pesticide exposure and occurrence of some persistent birth defects, our study of a group of DS patients from Western Gujarat also supports the same.

Our study further demonstrated that occupation like diamond polishing and agriculture might be associated with the higher risk of incidence of DS in these patients. These occupations are strongly associated with parental exposure to hazardous chemicals and environmental toxicants such as pesticides used in agriculture. Contrary to the existing belief in case of DS births, it was observed that there was no association or correlation between advanced parental age and the risk of occurrence of DS in offspring which may indicate that environmental and lifestyle factors may play a crucial role in causing nondisjunction at a younger age. As the occurrence of DS was independent of parental age, the occupational and environmental exposures might have possibly played a pivotal role in increased risk. The effect of exposure to pesticides in case of those individuals having farming related work is a possible explanation that environmental exposure might have played a role.

We further investigated the phenotypic characteristics of patients with DS and correlated these anatomical variations with physiological impairment of functions. Among clinical features, thyroid disorder was reported in 32% of the cases, vision related anomaly (24%), CHD (21%), and the digestive disorder (12%). Among the rest, skin, ear and hearing related abnormalities occurred in an equal number of patients.
Moreover, the parental and meiotic origin of trisomy 21 was assessed by QF-PCR using STR markers. Stochastic measures show recombination profile changes as the women ages however our examination proposed that there is no clear relation among meiotic error and the maternal age. Majority of the meiotic errors were of MMI type with maternal age 24.50±4.17 years and of MMII type with maternal age 27.78±3.0 years. Our results are in accordance with previous finding in the sense that most of the former studies reported that major etiology for trisomy 21 is maternal meiotic error followed by paternal age and mitotic origin. Additionally, the incidence of MMI error is higher than MMII error. Meiotic errors observed in this study were predominantly linked with young parents i.e. 101 out of 105 were under 33 years. As per our knowledge, there is any report describing occurrence of different meiotic errors and parental origin in DS cases from Gujarat. This remark recommends the need of additional research as what we observed are generally parents of young age. Prolonged exposures including radiations, everyday life (consumption of alcohol and tobacco), pesticides/insecticides, and professional exposures may cause errors during meiosis. Majority of our samples were belongs to farmers from poor economic background and reported to have use of pesticides for several generations. Thus, the current findings propose that there is a strong connection between the occupational factors and exposure to pesticides as a risk of DS. In addition, coming from a poor family background may subject them to malnutrition along with stress.

In summary, this study documented an interesting finding where environmental and lifestyle factors played the key role subduing the well-established parental age factor in the risk of occurrence of Down syndrome in offspring. Hence, like other genetic conditions, DS is expected to be the outcome of genetic, epigenetic, environmental and stochastic origin making it complex to discriminate their individual contribution.

**Part-B: Epigenetics study in Congenital Heart Defects (CHD)**

Congenital heart defects is the most common birth defect. Epigenetics refers to heritable transformations in gene expression without changing the nucleotide sequence. Our goal was to elucidate the epigenetic mechanisms involved in the development of non-syndromic CHD. Evidence suggests an important role of epigenetics in the development of CHD.
Methylation changes in heart development genes have been reported in cardiac DNA of CHD fetuses. A marked excess of de novo mutations in histone modification genes was observed in a large cohort of severe CHD cases. Neural crest stem cells, required for conotruncal development, are epigenetically regulated. Finally, microRNAs (miRNA), small non-coding, highly conserved post-transcriptional negative regulators of gene expression, are implicated in both normal cardiac development and CHD including genes regulating right outflow tract and ventricular septal development.

Our hypothesis was that epigenetic modification is an important mechanism in CHD pathogenesis through modulation of the transcription of gene networks involved in cardiogenesis. Our studies using genome-wide DNA methylation scans (GWMs) of blood and placental DNA in newborn CHD cases identified significant epigenetic changes in known cardiac developmental genes in different types of non-syndromic CHDs.

**B.1 Genome-wide methylation analysis in VSD**

Our studies using genome-wide DNA methylation scans (GWMs) of blood and placental DNA in newborn CHD cases identified significant epigenetic changes in known cardiac developmental genes in different types of non-syndromic CHDs. VSD the most common congenital heart defect, is characterized by a hole in the septum between the right and left ventricles. The pathogenesis of VSD is unknown in most clinical cases. There is a paucity of data relevant to epigenetic changes in VSD. The placenta is a fetal tissue crucial in cardiac development and a potentially useful surrogate for evaluating the development of heart tissue. To understand epigenetic mechanisms that may play a role in the development of VSD, genome-wide DNA methylation assay on placentas of 8 term subjects with isolated VSD and no known or suspected genetic syndromes and 10 unaffected controls was performed using the Illumina HumanMethylation450 BeadChip assay. We identified a total of 80 highly accurate potential CpGs in 80 genes for detection of VSD; area under the receiver operating characteristic curve (AUC ROC) 1.0 with significant 95% CI (FDR) p-values < 0.05 for each individual locus. The biological processes and functions for many of these differentially methylated genes are previously known to be associated with heart development or disease, including cardiac ventricle development (HEY2, ISL1), heart looping (SRF), cardiac muscle cell differentiation (ACTC1, HEY2), cardiac septum development (ISL1), heart morphogenesis (SRF, HEY2, ISL1, HEYL), Notch signaling
pathway (\textit{HEY2, HEYL}), cardiac chamber development (\textit{ISL1}), and cardiac muscle tissue development (\textit{ACTC1, ISL1}). In addition, we identified 8 microRNAs that have the potential to be biomarkers for the detection of VSD including: miR-191, miR-548F1, miR-148A, miR-423, miR-92B, miR-611, miR-2110, and miR-548H4. To our knowledge this is the first report in which placental analysis has been used for determining the pathogenesis of and predicting VSD.

Our study does have some limitations. One of them is the small sample size. We are now unable to evaluate the role of potential cofounders e.g. diet, obesity, and ethnicity on the methylation patterns. Our study, however, is a proof of concept study which generates plausibility data leading for more extensive analysis in a larger study population. Although it is generally recognized that DNA methylation correlates with gene expression, we did not test this correlation in our study. This is clearly an area for further study.

\textbf{B.2 Newborn blood DNA methylation-based biomarkers and key signaling pathway genes in TOF}

Tetralogy of Fallot is another common critical congenital heart defects (CCHD), requiring surgical or medical treatment within the first year of life. However, in majority of case the etiology of TOF is poorly studied. Our preliminary data as well as other research group showed that epigenetic changes may play crucial role in CHD. Epidemiologically, a large proportion of CHD including TOF fail to be diagnosed in the prenatal and early newborn period leading to health issues. To help find out the pathogenesis of TOF and identify potential molecular biomarkers for TOF, we performed genome-wide methylation assay in newborn blood in 24 non-syndromic TOF cases and 24 unaffected matched controls. Out of 64 CpG sites in 64 genes, many of these CpG loci are in genes that are already reported or suspected to be involved in CHD development or postnatal cardiovascular disorders. The CpG methylation difference between TOF and controls was \textasciitilde10\% in 51 CpG targets suggesting biological significance. Gene ontology analysis identified significant biological processes and functions related to these differentially methylated genes, including: CHD development, cardiomyopathy, diabetes, immunological, inflammation and other plausible pathways in CHD development. Multiple genes known or plausibly linked to heart development and post-natal heart disease were found to be differentially methylated in the
blood DNA of newborns with TOF including: *ABCB1, PPP2R5C, TLR1, SELL, SCN3A, CREM, RUNX and LHX9*. We generated novel and highly accurate putative molecular markers for TOF detection using leucocyte DNA and thus provided information on pathogenesis of TOF. Together with these reported genes, in this study we also identified some novel genes associated with TOF and CHD however these biomarkers require additional research.

Blood leucocytes could be modified epigenetically as they circulate through an affected primary organ. Our earlier studies suggested that leucocyte DNA might reflect cardiac epigenetic changes in CHD including TOF. Similarly this study also put forward a novel, non-invasive and potentially valuable move towards for the study of cardiac development and CHD. A blood based analysis found noteworthy methylation differences at CpG sites of multiple genes formerly known or possibly involved in cardiac development and post-natal heart disease such as TOF. We produced novel and precise putative molecular biomarkers for TOF. In future, these biomarkers could have application for TOF detection using leukocyte DNA.

The use of epigenetics to understand the mechanisms of heart defects is in its relative infancy and promises to help advance our understanding of these malformations. The identification of the causative mechanisms in CHD will not only improve understanding of disease mechanisms but could in the future contribute to the development of disease prophylaxis and therapy.

The present study provides new target genes and cellular pathways potentially involved in TOF development based on DNA methylation analysis based altered DNA methylation analysis. Although not definitive, our results highlight the potential importance of epigenetics in the pathogenesis of TOF. Finally, cardiac tissue is largely inaccessible in living fetuses and children so analysis using surrogate tissue such as blood could dramatically change our ability to detect and evaluate CHD.