

Original Research Article

Genetic study in congenital heart defects

Syed Abir Hussain^{1*}, Nasir u din Wani¹, Tasneem Muzaffar¹, Abdul Majeed Dar²,
Mohammad Akber Bhat², Roohi Rasool³, Imtiyaz Ahmad Bhat³, Shyam Singh²

¹Department of Cardio-Vascular and Thoracic Surgery, Government Medical College, Srinagar, Jammu and Kashmir, India

²Department of Cardio-Vascular and Thoracic Surgery, ³Department of Immunology, SKIMS, Soura, Srinagar, Jammu and Kashmir, India

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***Correspondence:**

Dr. Syed Abir Hussain,

E-mail: dr.syedabir@gmail.com

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ABSTRACT

Background: Congenital heart diseases (CHD) are relatively common with a prevalence ranging from 3.7 to 17.5 per 1000 live births. Little is known about genetic link with respect to congenital heart disease. Iroquoise (Irx) homeobox genes have been widely studied and their expression in both developing and adult heart. Author tried to study the role of irx4 and irx5 genes in structural congenital heart disease, keeping the focus on study reported by Cheng Z et al.

Methods: Author studied reported mutation site sequences in 25 various congenital heart disease patients and control healthy relatives of patients. It is a unique study and there has not been such a study reported in literature till date. Besides comparison with healthy related controls, author took cardiac tissue biopsy in patients while doing corrective cardiac surgery. However, blood samples were taken from controls due to ease of feasibility.

Results: Although, there were no sequence variations in the studied exon regions, but author got a base pair sequence change at 6 bp intron region, which is near the exon splice site in irx4 gene. Besides two ASD patient's male children (one child each) had ASD prompting us to believe some role of sex linkage. However later needs pedigree analysis and sex chromosome studies for further analysis.

Conclusions: Gene sequence in the Kashmiri population is unique. There is possibility of role of irx genes in CHD. ASD might have sex linkage in some.

Keywords: Congenital, Heart disease, Genes Irx4, Irx5

INTRODUCTION

Congenital heart diseases (CHD) are relatively common with a prevalence ranging from 3.7 to 17.5 per 1000 live births.^{1,2} However, in India a higher prevalence (26.3 per 1000 live births) has been reported.³ Ventricular septal defect (VSD) is the most common among acyanotic CHD and Tetralogy of Fallot the commonest among cyanotic. Congenital heart defects may be broadly grouped into

two major categories which were morphological abnormalities including developmental defects resulting in structural malformations and functional abnormalities including cardiac rhythm disturbances and cardiomyopathies.

It is very important to determine whether there is an underlying genetic pattern for the reasons which were may be other important organ system involvement, there

may be prognostic information for clinical outcomes, there may be important genetic reproductive risks the family should know about and there may be other family members for whom genetic testing is appropriate.

Mammalian heart development begins with the specification of cardiac progenitor cells within the anterior lateral plate mesoderm.^{4,6}

Iroquois homeobox (Irx) genes were first discovered in *Drosophila melanogaster*, in which mutations of the Iroquois genes resulted in loss of bristle formation on the lateral notum (dorsal mesothorax), leaving only a band of bristles in the median part of the notum, resembling “Mohawk”- hairstyle of the Iroquois tribes of Native Americans-from which the locus name is derived.^{7,8} Irx gene family members encode highly conserved homeodomain-containing transcription factors (TFs) and play fundamental roles in diverse developmental processes in both invertebrates (*Drosophila*) and vertebrates.^{9,10} It is pertinent to point out here that homeobox is a highly conserved region of 180 base pairs in these genes that specifies a highly conserved corresponding 60 amino acid domain segment called homeodomain.¹¹ Unlike typical DNA binding homeodomain (HD) transcription factors, which contain 60 amino acid residues with 3 alpha helices, 12 Irx proteins contain an atypical HD with 3 extra amino acids between the first and second alpha helices, thus placing them in the 3-amino-acid-loop-extension (TALE) family of transcription factors (TF).¹³ Irx TFs also have a conserved motif (Iro-box) of 13 amino acid residues in the carboxyl-terminal region, whose function has not been established yet.^{14,15} *Drosophila melanogaster* expresses 3 closely related proteins, araucan (ara), caupolican (caup), and mirror (mirr), organized in a gene cluster called the Iroquois complex (Iro-C). Mammals including humans have 6 Irx genes clustered in two 3-gene groups; the IrxA cluster on mouse chromosome 13 (human chromosome 5) contains Irx1, Irx2, and Irx4, and the IrxB cluster on mouse chromosome 8 (human chromosome 16) consists of Irx3, Irx5 and Irx6.

METHODS

The study was conducted in the Departments of Cardio-Vascular and Thoracic Surgery and Department of Immunology of Sher-I- Kashmir Institute of Medical Sciences (SKIMS), Srinagar, Jammu and Kashmir, India.

A total of 25 congenital heart disease patients were included in the study. Blood samples from 25 healthy relatives of patients, who were considered as controls, were also taken for Irx 4 and Irx 5 gene study. A written informed consent was obtained from all cases and controls. Author selected matched controls from family members, preferably first-degree relatives.

Data on all cardiac patients were obtained by personal interviews. The data collected included gender, age,

family history of congenital heart disease etc. The collection and use of tissue and blood samples for this study has been approved by the Institute Ethical Committee.

The tissue specimens from right atrial appendage (Figure 1 and 2) resected during surgical management of the disease were collected from 25 congenital heart disease patients for the study. All tissue and blood specimens were immediately stored at -70°C for nucleic acid extraction. Tissue samples were used for mutational analysis of IRX 4 and IRX 5 genes.

In addition, blood was also collected from 25 healthy random cases. 5 ml of peripheral blood was obtained from each subject in EDTA containing vials (200 µl of 0.5M, pH=8.0) and stored at -20°C. The blood samples were also used for mutational analysis of IRX 4 and IRX 5 genes.

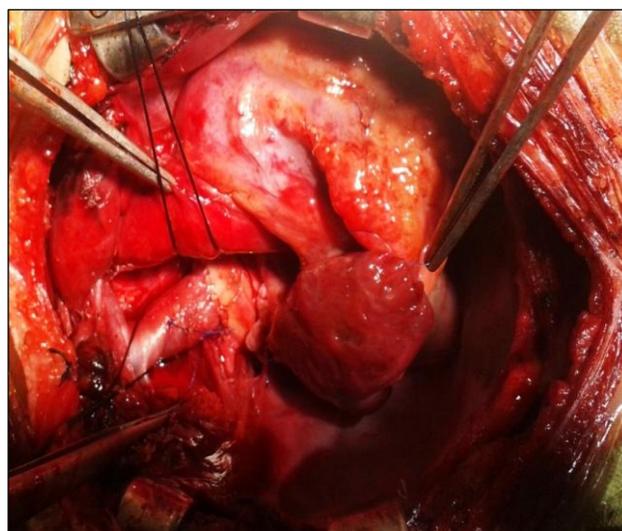


Figure 1: Biopsy taking from right atrial appendage in a patient operated for Glen shunt.

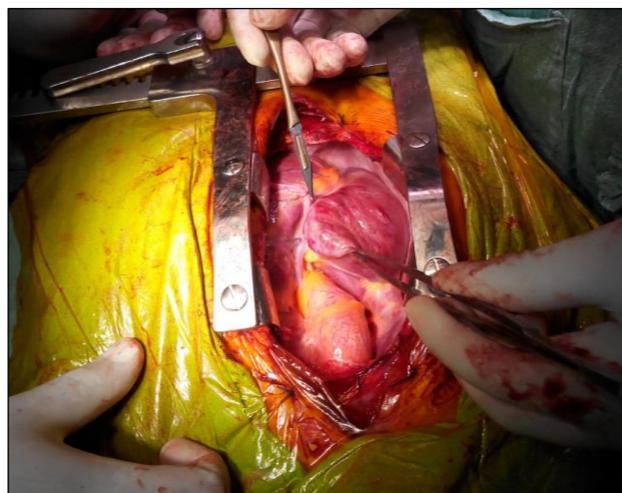


Figure 2: Biopsying right atrial appendage.

High-molecular-weight DNA was isolated (Figure 3) from tissue and blood samples using phenol-chloroform proteinase K and/or ammonium acetate method.¹⁶

The quality of the DNA obtained from the tissue specimens and blood samples was analyzed on 1% agarose gel. Two sets of primers (Table 1) were used for amplification of exons Irx4 and Irx5 (Figure 4 and 5).

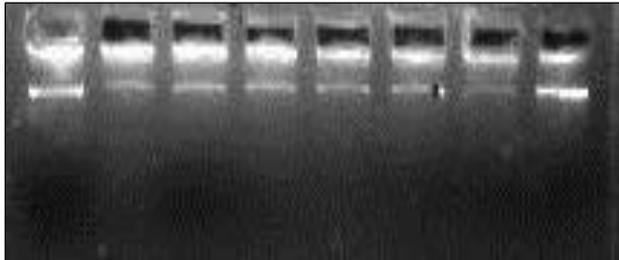


Figure 3: Genomic DNA isolated from blood, tissue. Lanes 1-8 were DNA extracted from different tissues/blood samples.

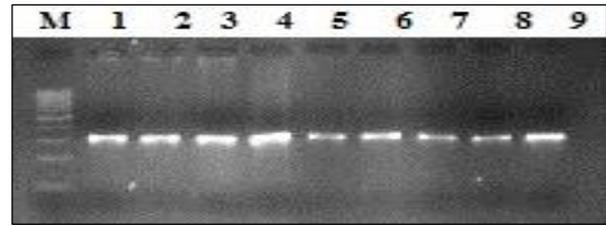


Figure 4: Representative amplification picture of Irx4 exon 3.

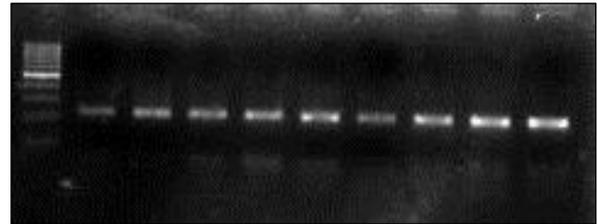


Figure 5: Representative amplification picture of Irx5 exon 2.

Table 1: Primer sequences and annealing temperatures of Irx 4 and Irx 5 genes for direct sequencing.

Gene	Exon	Primer Sequence	T _m (°C)	Product size (bp)
Irx 4	3	F: 5'-GGTCTACTGCCCGGTCTA-3' R: 5'-CAAAGTTGAAGGCAGCCAAG	60	299
Irx 5	2	F: 5'-AAC GAG CAC CGC AAG AA R: 5'-GTC GTT CTT CTC CAG GTC AAT	60	239

F= forward primer, R= reverse primer, T_m = annealing temperature, bp= base pair.

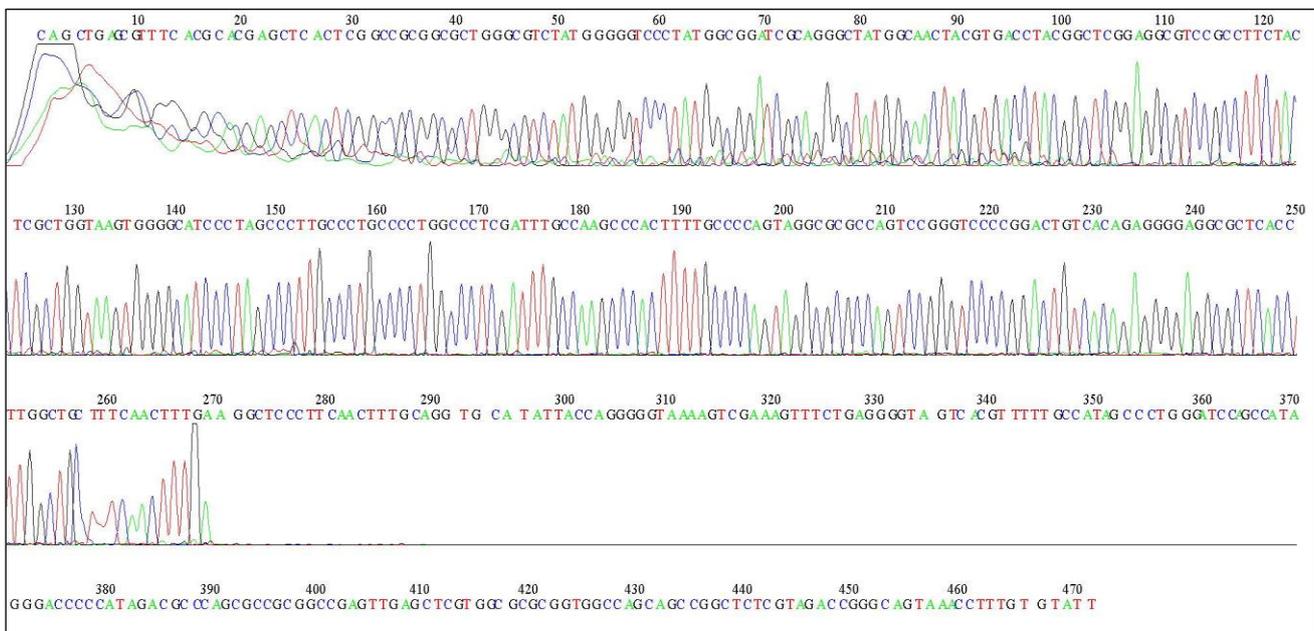


Figure 6: Irx4 sequencing normal position of T (Thymine) between 130 and 140 base pair regions.

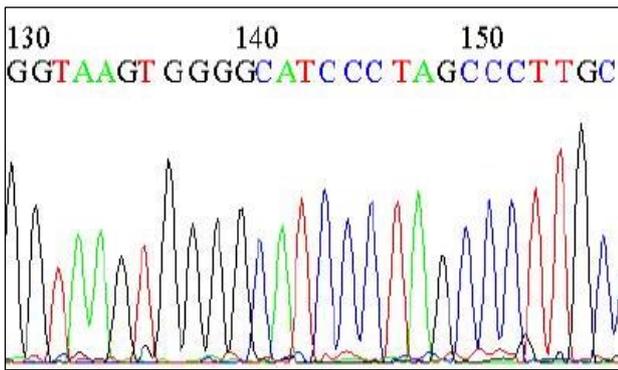


Figure 7: Irx4 sequencing picture normal position of T (Thymine) between 130 and 140 base pair regions (Zoomed view).

DNA sequencing was carried out by automated DNA sequencing method on ABI Prism 310 (Perkin Elmer) (Figure 5, 6, 7, 8, 9 and 10). The automated DNA sequencing is based on the chain termination reaction.

Genotypic and allelic distributions were compared using the chi-square test. Continuous variables were compared with a two tailed t-test and expressed as means±standard deviation (SD), while categorical variables were compared using chi-square test.

A p value of less than 0.05 was considered statistically significant. All statistics was done using SPSS software (Windows, version 20.0).

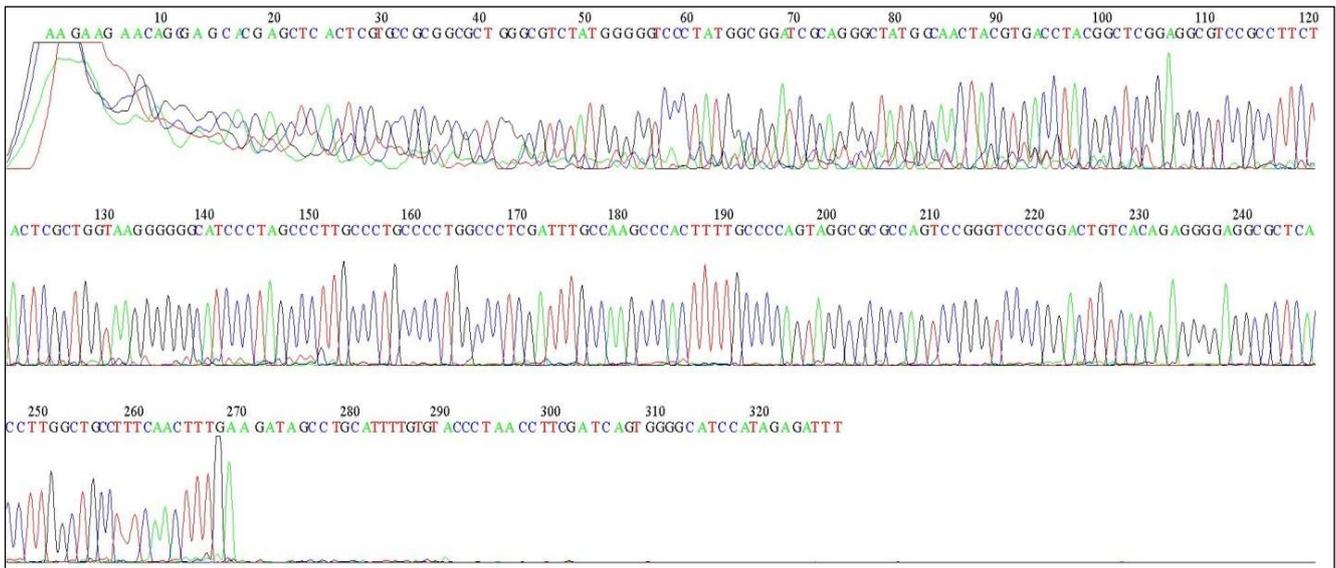


Figure 8: Irx 4 gene sequencing picture base G replacing T (between 130-140).

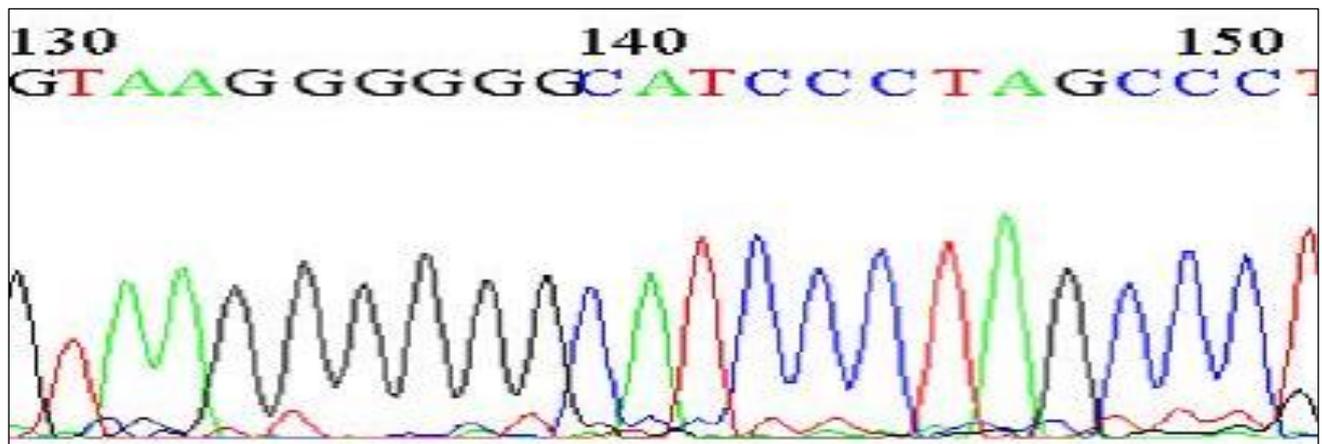


Figure 9: Irx 4 gene sequencing picture base G replacing T (between 130-140) in zoomed view.

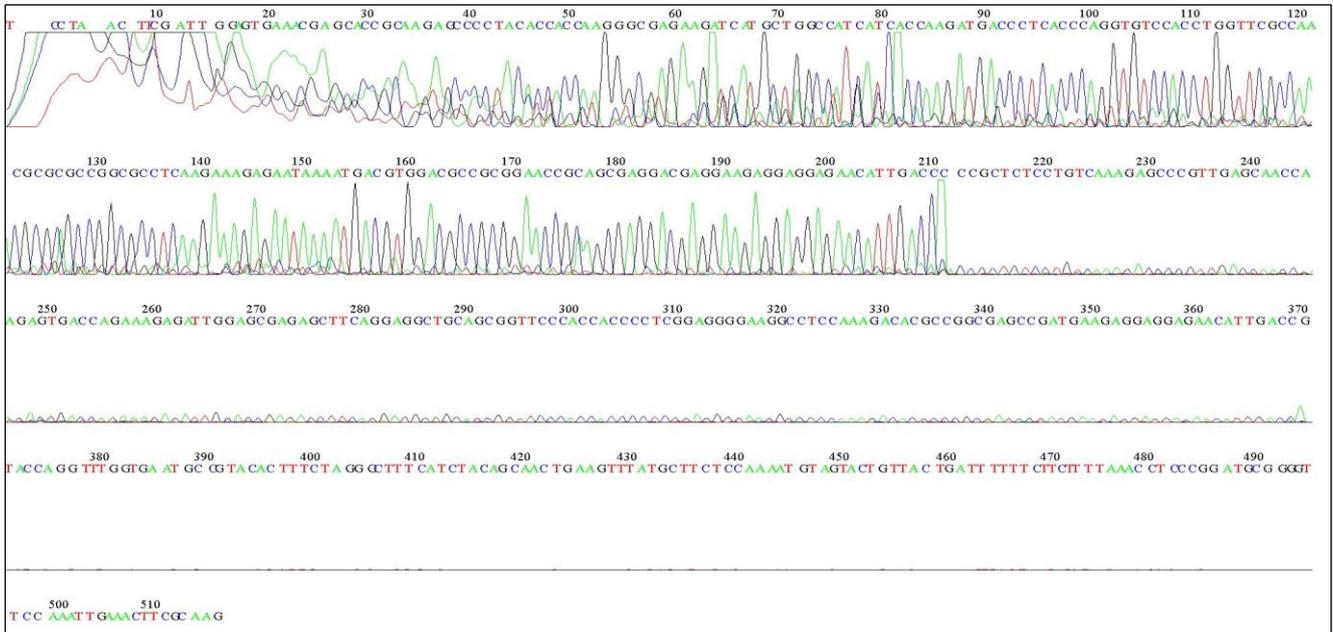


Figure 10: Irx 5 sequencing picture normal base pair sequence.

RESULTS

The age in patients varied between minimum of 3 years and maximum of 46 years with a mean of 22.68 and standard deviation of 12.02.

Age in the controls varied between minimum of 5 years and maximum of 48 years with a mean of 23.77 and standard deviation of 10.78.

There were 13 (52%) females and 12 (48%) males in the patient group and 11 (44%) females and 14 (56%) males in the control group. There was significant family history in two female ASD patients with one male child having ASD secundum (ASD 2) each. One more male ASD secundum patient had family history of rheumatic heart disease in one female sib. 8 (32%) patients in this study had class 1, 15 (60%) had class 2 and 2 (8%) had class 3 symptoms. None of the patients had class 4 symptoms. Out of 25 patients, 13 (52%) patients had uncomplicated ASD secundum (ASD 2), 1 (4%) had ASD secundum with pulmonary stenosis, 1 (4%) had ASD secundum with mitral valve prolapsed, 1 (4%) ASD secundum with LSVC, 1 (4%) superior venacava type sinus venosus ASD, 1 (4%) partial atrio-ventricular canal defect or septum primum defect, 1 (4%) tricuspid atresia (TA), 1 (4%) tetralogy of Fallot (TOF), 1 (4%) sub-aortic membrane (SAM), 2 (8%) perimembranous VSDs and 2 (8%) had bicuspid aortic valves.

About 10 (40%) patients underwent respective ASD secundum closure via anterolateral thoracotomy (ALT) approach, and on beating heart. 2 (8%) patients had median sternotomy with closure of the atrial septal defect

on beating heart which include a Glen shunt performed off cardiopulmonary bypass.

About 12 (48%) patients had median sternotomy with procedure completed under cardiac arrest using standard cardioplegia.

Around 1 (4%) patient underwent ALT with closure of the atrial septal defect under cardiac arrest.

Total 8 (32%) defects were closed using pericardial or PTFE patch including 2 VSD, 4 ASD, and one TOF in whom intracardiac correction was done using PTFE patch. In SAM patient, the sub-aortic membrane was excised under cardiopulmonary bypass via aortotomy. Two bicuspid aortic valve patients had aortic valve replacement by conventional approach.

The mutational analysis of Irx 4 (exon 3) and Irx 5 (exon 2) sequence was done. Although no mutation was seen in these exons however, author found a novel change at 6 splice site T>G (T-thymine replacing G-guanine) in the Irx4 gene in the intronic region in this studied group (Figure 8 and 9). The electropherograms showing a change at 6 splice sites is given below. On manual reading of the sequence in Irx4, author could group change as homozygous TT= 2, homozygous GG= 2, heterozygous GT= 21 in cases and TT= 3, GG= 2, GT= 20 in controls (Table 2).

Irx 5 exon 2 mutational analysis in 25 patients and 25 controls revealed normal sequence in all the patients/controls and no abnormal sequence was observed in these (Figure 10).

Table 2: Irx 4 exon 3 tabulation regarding 6 splice site sequence as inferred from sequencing diagram, homozygous (TT, GG)/heterozygous (GT).

Base pair	TT	GG	GT
Patients	2 (8%)	2 (8%)	21 (84%)
Control	3 (12%)	2 (8%)	20 (80%)

DISCUSSION

Congenital heart defects (CHD) represent a significant proportion of patients in all races. A lot of progress has been made over past few decades in understanding these defects and their management. At present there is hardly any such defect for which there is no treatment available, palliative or curative. However little albeit significant progress has been made in understanding their genetic basis and the effect of various gene mutations related to heart development.

In the studied samples, no change was observed at codon 85 and 92 as has been reported by Cheng Z et al, in two Chinese VSD patients.¹⁷ Animal studies have shown that Iroquois genes are expressed in a specific pattern during heart development. Many gene mutations have been reported from various studies in association with CHD viz. *nkx2.5*, *GATA*, *FOG2* etc.¹⁸⁻²⁰ One such study was conducted by Cheng Z et al, on 698 Chinese patients regarding *Irx4* gene.¹⁷ They reported two mutations in two VSD patients in two different locations. They further did functional analysis in these cases which showed that the mutation affected the interaction between *Irx4* and *RXR α* . These findings indicated that the mutated proteins showed weakened protein-protein interactions. They concluded with a note of causative role of these mutations. However, they investigated the germline mutations only as they used blood samples in their study. Present study is its kind of own. Author have used cardiac tissue properly preserved at -70°C. Thus, there was a more probability of getting positive results. However, because of financial constrains sample size in this study was small, as compared to the reference study of Cheng Z et al.¹⁷

Out of 25 patients there was significant family history of cardiac disease in first degree relative. This could be seen in two ASD secundum patients. One male patient whose mother was earlier operated in the same hospital for ASD secundum closure and a female patient whose son also had ASD secundum. This could raise the suspicion of X-linked phenomenon in our minds. The further study in these families, needs pedigree analysis for the same which has not been performed yet. There was family history of Rheumatic heart disease in sister of another male ASD secundum patient. Benson D et al, report three families with 9 of twenty family members affected with ASD.²¹ Familial ASD is a genetically heterogeneous disorder, one disease gene maps to chromosome 5p. Recognition of the heritable basis of familial ASD is

complicated by low disease penetrance and variable expressivity.

Mutational analysis of collected samples i.e. tissue mainly from patients and blood from controls revealed no mutation in reported mutation-cluster regions of *Irx4* and *Irx5* genes in either group. However, author found a novel change at 6 splice sites in intronic region of *Irx4* gene, wherein guanine (G) got replaced by thymine (T>G) at 6840. On manual reading of the sequence in *Irx4*, author could group the change as homozygous TT=2, homozygous GG=2, heterozygous GT=21 in cases and TT=3, GG=2, GT=20 in controls. Whether this change is a mutation or polymorphism remains to be seen. Bayrak F et al, studied *Irx4* gene in 68 hypertrophic cardiomyopathy patients and report four polymorphisms which include G355>A, A381>G, G1203>A and C1431>T.²²

Population studies in healthy unrelated controls are under way for the same. Association with congenital heart disease patients of such a change may have impact during gene expression as it is close to the gene splice site. This requires further large scale studies. However, author are sure that this change might have significance because of its high frequency in the studied population which comprised of patients with CHD and their relatives.

CONCLUSION

Although, present study with *Irx4* and *Irx5* genes did not yield direct correlation with congenital heart disease, however base pair abnormalities at 6 splice sites in *Irx4* intron could raise the possibility of some interference in gene functioning. Besides X-linked clue in two ASD secundum patients was also seen. However, small sample size and lack of pedigree analysis warrants need for further studies.

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Conflict of interest: None declared

Ethical approval: The study was approved by ethical committee of Sher-i-Kashmir Institute of Medical Sciences, Soura, Srinagar, Jammu and Kashmir, India

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