

ANALYSIS OF GENE COPY NUMBER VARIATIONS IN PATIENTS WITH CARDIAC SEPTAL DEFECTS USING MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION

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ABSTRACT

Objective: Cardiac septal defects (CSDs), the most common human congenital heart malformations are complex and heterogeneous. Progress in molecular biology has helped to identify many genes responsible for cardiac morphogenesis. However, etiologic factors in familial as well as isolated syndromes are being identified; the root genetic cause still needs to be resolved and its mechanism is yet to be revealed. The objective of this study is to identify DNA copy number variations (CNVs) and their possible association with septal defects.

Methods: Multiplex ligation-dependent probe amplification (MLPA) was used to detect DNA copy number in non-syndromic CSDs using the P311-A1 Kit consisting of probes for the key genes, namely, NKX2-5 (NK2 transcription factor related, locus 5), GATA4 (GATA binding protein 4), TBX5 (T-box transcription factor), bone morphogenetic protein 4, and CRELD1 (cysteine rich with EGF-like domains 1).

Results: We studied 124 clinically diagnosed CSD subjects, of which 111 (89.5%) had atrial septal defects and 13 (10.5%) had ventricular septal defects. MLPA assay was carried out in all these patients after a thorough clinical and cytogenetic screening. CNVs were identified in 16 (12.9%) cases, of which heterozygous deletions and heterozygous duplications were detected (8 patients each) with apparent phenotypes.

Conclusion: MLPA could be a useful assay for the detection of CNVs and to be adopted as the first line of screening in patients with congenital heart diseases.

Keywords: Copy number variations, Atrial septal defects, Ventricular septal defects, Multiplex ligation-dependent probe amplification, Heterozygous deletions, Heterozygous duplications.

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INTRODUCTION

Cardiac septal defects (CSDs) constitute 1% of all congenital heart diseases (CHDs) [1]. The prevalence of CHD worldwide (population based) is between 1 and 150/1000 live births, while in India, it is 8.07/1000 live births [2]. However, septal defects have a reported frequency of 4.1/1000 live births. CHDs can either be isolated (non-syndromic; septal defects) or in combination with other cardiac lesions (syndromic; Holt-Oram syndrome). The septal defects such as atrial septal defects (ASDs) and ventricular septal defects (VSDs) may be asymptomatic, with the appearance of murmur as the primary symptom. Many small septal defects close on their own as the heart grows; hence, percentage diagnosis that is typically made during childhood is low, except for a few which persist through adulthood. The diagnosis is usually based on echocardiography and transesophageal echocardiography. Congenital septal defects are the most common of all structural malformations of the human heart and are caused by both genetic and environmental factors [3]. Since the year 2000, several genetic epidemiological studies have confirmed its genetic predisposition [4]. Subsequent advancements in the molecular diagnostics helped in identifying genes responsible for cardiogenesis and causative of syndromic and non-syndromic forms of CHDs. Transcriptional factor genes are predominantly articulated in the heart tissue and are documented to have a physical interaction with other regulatory proteins for proper morphogenesis [5]. Despite recent molecular genetic studies, efforts to identify genotype-phenotype correlation in patients with septal defects suggest a more complex pattern of

inheritance, where copy number variations (CNVs), as well as single nucleotide polymorphisms, contribute to the disease pathogenesis and tune the spectrum of septal malformations. CNVs contribute majorly toward the genetic diversity in context to evolution and disease susceptibility. Therefore, it is essential to detect the CNVs associated with CHDs. In this study, a multiplex ligation-dependent probe amplification (MLPA) assay was used to find out copy number changes in a cohort of 124 non-syndromic patients with CSDs. This MLPA assay is mainly used to utilize the many advantages provided when compared to other techniques. In fact, this assay allows a pool of samples to be handled simultaneously so that it could be used in high throughput analysis to find out copy number changes. MLPA could be a promising technique for proper diagnosis, prognosis, and management of the patients and affected families.

METHODS

In this study, the patients were referred from the outpatient cardiology department of Kasturba Hospital, Manipal. Subjects were ascertained through echocardiography to confirm the diagnosis and the size of the septal defects. The patient's details were noted in a case proforma which includes parental age at pregnancy, parental consanguinity, maternal gestational history, family history, history of any occupational/environmental exposures, socioeconomic status, and a three-generation pedigree. A written informed consent was obtained from the patient/guardian. Both the cardiologist and the genetic counselor analyzed the assessment of clinical phenotypes. In patients with CNVs, clinical and laboratory evaluations of the parents were included in the study.

Peripheral blood samples (5 ml) were collected from each participant to conduct both cytogenetic and MLPA analysis. GTG-banded karyotype (≥ 450 bands) was performed according to the modified method [6]. Lymphocytes were cultured and stimulated with known mitogen (Phytohemagglutinin) for 72 h. Metaphase was arrested with colchicine and cells harvested using 0.075 M KCl and Carnoy's mixture (methanol/acetic acid [3:1]). The slides were stained, and metaphases were captured, and analysis was done using a Zeiss microscope and IKAROS software.

Genomic DNA was extracted from whole blood of recruited patients (n=124) as well as controls (n=17; 8 males and 9 females) using classic phenol-chloroform and QIAamp[®] genomic DNA kit (QIAGEN) according to the manufacturer's instructions. MLPA assay was performed as per the manufacturer SALSA MLPA kit P311-A1 (MRC-Holland, Amsterdam, Netherlands) protocol. Hybridization, ligation, and amplification were performed as described by Schouten *et al.* [7]. Two microlitre of the amplified product were diluted (1:30) in 58 μ l of distilled water, of which 2 μ l was plated into a well with 30 μ l of a mixture of 1 part of formamide Hi-Di (Applied Biosystem) and 0.015 parts GeneScan 500 LIZ size standard (Applied Biosystem). Capillary electrophoresis was performed on AB 3130 DNA analyzer (Applied Biosystems) as per the standardized laboratory protocol.

GeneMapper software v4.0 (Applied Biosystem) was used to perform fragment sizing with the internal 500 LIZ size standard, automated peak calling, and peak normalization. Thirty-five peak heights of 35 probes (linked to GATA4, NKX2-5, TBX5, BMP4, CRELD1, and DGCR8 genes) of each sample were exported for further data analysis. The peak heights were normalized by dividing each peak height by the sum of all 35 peak heights in each sample. Each normalized peak height was divided by the means of that peak height in the control probe. A spreadsheet was developed in Microsoft Excel to process the data for the control samples efficiently.

RESULTS

A total of 124 subjects (54 males and 70 females) with septal heart defects, aged between 3 (children) and 65 years (adult) registered at Cardiology OPD, Kasturba Hospital, Manipal. A case proforma was maintained to gather the clinical and family history which was used to analyze the correlation data. VSDs are the most common of all congenital cardiac anomalies, whereas in our study, ASDs were more common, due to a higher number of adult patients. The clinical phenotypes of these subjects are ASDs (n=111) (Ostium secundum [OS]-ASD [n=102] and sinus venosus [SV]-ASD [n=9]) and VSDs (n=13) (perimembranous

[Pm]-VSD [n=7], muscular VSD [n=1], subpulmonic VSD [n=1], subaortic VSD [n=1], and infravalvular gerbode VSD [n=3]). Familial recurrence was observed in 6/124 patients, whereas consanguinity was seen in 18/124 patients. Nearly 54.40% of the patients showed breathlessness and 58.4% had fatigue. A two-dimensional echocardiography was done in all patients; gradient and size of the septum were noted accordingly. Echocardiography revealed the thickness of either atrial or ventricular septum to be between <10 mm and 50 mm (<10 [n=40; 32.2%], 11 and 20 [n=49; 39.5%], 21 and 30 [n=31; 25.0%], 31 and 40 [n=3; 2.41%], and 41 and 50 [n=1; 0.80%]). Family history and cardiac profiles such as electrocardiography, systolic functions, auricle dilation, right ventricular systolic pressure, and pulmonary arterial hypertension were also recorded. A cytogenetic study was carried out in every recruited patient as per the inclusion criteria to rule out gross chromosomal abnormalities and syndromes, and only isolated cases were included in the detection of CNVs.

MLPA analysis was done using the P311-A1 CHD SALSA MLPA Kit. Controls were run in the ratio 1:7 to the subject samples. A total of 16 (12.9%) cases was found to have copy number changes in NKX2.5, GATA4, TBX5, BMP4, and DGCR8, as shown in Table 1. Heterozygous deletions were observed in eight patients and heterozygous duplications were observed in eight subjects with apparent phenotypes. The fragment separation was done using capillary electrophoresis, which employed an ABI 3130 sequencer. The fragment analysis was performed using a trial version of Gene Marker software from SoftGenetics. The regions covered in the MLPA reactions using SALSA MLPA Kit P311-A1 are MSR1, CTSB, CRELD1, GATA4, TBX5, NKX2.5, GP1BB, BMP4, DGCR8, and CDC45 genes.

Sixteen CNVs (eight heterozygous deletions and eight heterozygous duplications) were identified in 124 patients studied with isolated CSD and the data are represented in Table 1. The following observations were derived from the data obtained. (1) The heterozygous duplication in exon 1 (5q35.1) of NKX2.5 was identified in an 18-year-old female with Pm-ASD phenotype; this patient had a family history of OS-ASD in both mother and elder sister. Interestingly, the same variation was detected with different phenotypes (OS-ASD) in two other patients, as shown in Table 1. (2) Heterozygous CNVs were found in five adult female patients, two cases of deletion in exon 1 (8p23.1) of GATA4 and three duplications in exon 6 of GATA4, of which four patients had OS-ASD while one had Pm-VSD. (3) Similarly, heterozygous CNVs in exon 9 (deletion) and exon 6 (duplication) in the TBX5 gene (12q24-22) were detected in four patients with OS-ASD in three and Pm-VSD in one patient. (4) In the DGCR8 gene (22q11.21), we detected

Table 1: Clinical phenotypes and heterozygous variants identified in 16 subjects with heart septal defects

S. No.	Age and gender	Clinical phenotype	Gene/chromosome position	GenBank exon	Results
1.	18/F	Pm-VSD	NKX2.5 (5q35.1)	1	Heterozygous duplication
2.	53/F	OS-ASD	NKX2.5 (5q35.1)	1	Heterozygous duplication
3.	66/F	OS-ASD	NKX2.5 (5q35.1)	1	Heterozygous duplication
4.	28/F	OS-ASD	GATA4 (8p23.1)	1	Heterozygous deletion
5.	50/F	OS-ASD	GATA4 (8p23.1)	1	Heterozygous deletion
6.	23/F	Pm-VSD	GATA4 (8p23.1)	6	Heterozygous duplication
7.	41/F	OS-ASD	GATA4 (8p23.1)	6	Heterozygous duplication
8.	51/F	OS-ASD	GATA4 (8p23.1)	6	Heterozygous duplication
9.	11/F	OS-ASD	TBX5 (12q24.22)	9	Heterozygous deletion
10.	36/F	OS-ASD	TBX5 (12q24.22)	9	Heterozygous deletion
11.	31/M	Pm-VSD	TBX5 (12q24.22)	6	Heterozygous duplication
12.	40/M	OS-ASD	TBX5 (12q24.22)	9	Heterozygous duplication
13.	9/M	OS-ASD	TBX5 (12q24.22) BMP4 (14q22.2)	4,9	Heterozygous deletion
14.	4/F	Pm-VSD	TBX5 (12q24-22) BMP4 (14q22.2)	4,9	Heterozygous deletion
15.	58/M	SV-ASD	DGCR8 (22q11.21)	14	Heterozygous deletion
16.	24/F	SV-ASD	DGCR8 (22q11.21)	14	Heterozygous deletion

F: Female, M: Male, Pm-VSD: Perimembranous ventricular septal defects, OS-ASD: Ostium secundum atrial septal defect, SV-ASD: Sinus venosus atrial septal defect, NKX2-5: NK2 Transcription factor related, locus 5, GATA4: GATA binding protein 4, TBX5: T-box transcription factor, BMP4: Bone morphogenetic protein 4, DGCR8: DiGeorge syndrome critical region gene 8

a heterozygous deletion in exon 14 in two patients with SV-ASD. (5) Two CNVs detected in this study were found to be associated with overlapping phenotypes, namely, the GATA4 (8p23.1) and the TBX5 (12q24-22) variations were found in patients with OS-ASD as well as Pm-VSD.

DISCUSSION

Septal defects can be caused by mutations in various transcriptional genes such as NKX2.5, GATA4, and TBX5. Synergic interactions of these genes are inevitable in cardiogenesis, particularly in the formation of chambers of the heart. Among mutations reported in these genes, CNVs including deletions and duplications are the most common in the general population with a frequency of 1–5% [8,9]. Sometimes, benign CNVs may disrupt the reading frame of the gene and cause premature termination, causing splice site defects or even exon skipping. Genomic CNVs are playing a pivotal role in genetic architecture and well documented in many of the congenital abnormalities including heart defects and neurodevelopmental disorders such as developmental and intellectual disabilities [10,11]. Increasing knowledge of CNVs has moved focus toward a better understanding of CHDs [12]. Numerous techniques such as quantitative fluorescence polymerase chain reaction (PCR), MLPA, microarray, and next-generation sequencing have been described to quantify CNVs in human genetic disorders. However, MLPA assay has become one of the most widely used techniques to detect CNVs in several genetic diseases [13,14]. Several studies have shown the presence of recurrent CNVs detected in the patients with syndromic and non-syndromic CHD, including Sorensen's group [15] through MLPA screening.

In the present study, we detected copy number changes in 16 subjects (12.9%) among 124 cases with septal defects who had a normal karyotype. Heterozygous deletions were identified in OS-ASD (n=5), SV-ASD (n=2), and Pm-VSD (n=1). Heterozygous duplications were identified in Pm-VSD (n=4) and OS-ASD (n=5). However, none of these variations was inherited from the parents. However, duplications and deletions were observed in association with the same phenotypes. In various studies [16,17], similar phenotypes have been observed, where they speculated the variable expressions of the TBX5 gene. However, Liu *et al.* [18] revealed no evidence of GATA4 and NKX2.5 CNVs in the fetal CHD of the Chinese population except for the deletion in 22q11. Whereas, Guida *et al.* [19] revealed that individuals with isolated CHD screened for CNVs in their cohort shown had mutation-negative for GATA4. This may be due to the variation in geographical location and ethnicity. However, we found deletions and duplications in NKX2.5, GATA4, TBX5, and BMP4, as shown in the results.

We identified one deletion (148nt) and one duplication (238nt) in GATA4 at chromosomal position 8p23.1 in three OS-ASD and one Pm-VSD patient. As per the Database of Genomic Variants catalogue control population, no CNVs have been found to overlap with GATA4. Therefore, it is expected to conclude that septal defective phenotypes in these four individuals with CNVs at 8p23.1 may have resulted from the dosage sensitivity of GATA4 [20]. 8p23.1 duplication in GATA4 with variable phenotype, expressivity, and penetrance has been found to be associated with septal defects, conotruncal heart defects, developmental delay, and mild dysmorphism. Altered GATA4 dosage may vary due to variations in the TBX5 or other genes [21]. Interestingly, we detected one duplication each in 12q24.21 of TBX5 (346nt) in one OS-ASD and one Pm-VSD patient. NKX2.5 duplication has been observed at 5q35.1 in two OS-ASD patients (274 nt) and one Pm-VSD patient. In the case of Pm-VSD, NKX2.5 duplications were detected at two different regions (190 nt and 274 nt). We also detected a BMP4 deletion (391 nt) at 14q22.2 in two patients: One with OS-ASD and another with Pm-VSD; these two cases also showed a deletion in TBX5 (303 nt). In this study, we detected a 22q11.21 deletion (382 nt) in the region DGCR8 in two patients with SV-ASD. A similar CNV was reported earlier by Liu's group [18] in a patient with Tetralogy of Fallot.

CONCLUSION

Septal defects are a complex trait as multiple factors have been implicated in their pathogenesis, but the etiology has a strong genetic component, as shown by extensive epidemiological studies. The present study derives an inference that heterozygous deletions/duplications cause the incomplete formation of the cardiac septum and may lead to structural defects. This may be attributed to the misregulation of messenger RNA and protein synthesis during embryonic heart development. We, therefore, hypothesize that the loss/gain of transcriptional activity may cause structural and functional defects in the postnatal heart. Here, MLPA was used for the detection of microdeletions/duplications, due to its advantages over PCR and array comparative genomic hybridization, concerning practical simplicity and speed. MLPA is easy to implement in a standard diagnostic laboratory. In addition, any genomic region associated with CHDs can be targeted specifically using MLPA.

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AUTHORS' CONTRIBUTIONS

Dr. Yashvanthi Borkar, Senior Research Fellow, preparation of the manuscript. Dr. Krishnananda Nayak, a technologist, provided the details of Echocardiography. Dr. Ranjan K Shetty, clinical cardiologist made the clinical diagnosis of every patient and reviewed the manuscript. Dr. Rajasekhara Moka supervised the manuscript preparation and review.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare in relation to this article.

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