International Journal of Medical Laboratory 2022;9(1):65-72.

Original Article

# CNV Analysis Using Multiplex Ligation-Dependent Probe Amplification in Iranian Families with Non-Syndromic Congenital Heart Defects: Early Diagnosis of Non-Syndromic Patients

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## ABSTRACT

***Article history***
Received: 19 May 2021
Accepted: 2 Jan 2022
Available online: 16 Mar 2022

***Keywords***
CNV
Familial CHD
MLPA
Non-syndromic CHD
Sporadic CHD

**Background and Aims:** Congenital heart defects (CHD) are the most common type of congenital disability. Copy number variations (CNVs) have been found as one of the genetic etiology of non-syndromic CHD, and researchers have detected several pathogenic CNVs in patients with cardiac defects.
**Materials and Methods:** In the present study, 70 patients with familial (20 patients) and sporadic (50 patients) non-syndromic CHD were evaluated to find whether CNVs in the *GATA4*, *NKX2-5*, *TBX-5*, *CREL*, *BMP4* genes, and 22q11.2 region contribute to the pathogenesis of non-syndromic CHD. We have used the Multiplex Ligation-dependent Probe Amplification (MLPA) technique as a molecular method to identify CNVs in predefined loci.
**Results:** Normal MLPA results were demonstrated for *GATA4*, *NKX2*-5, *TBX*-5, *CRELD*, and *BMP4* genes for all sporadic and familial cases. However, we found three patients with imbalances for the 22q11.2 region. One patient with 22q11.2 deletion showed tetralogy of fallot, and the other had ventricular septal defects/ pulmonary atresia/ multiple aortopulmonary collateral arteries. A duplication of the 22q11.2 region was detected in one patient with patent ductus arteriosus.
**Conclusion:** Identifying genomic imbalances in 6% of the non-syndromic sporadic patients indicates that recurrent CNVs could be associated with non-syndromic CHD. It seems that it is the first CNV analysis using MLPA carried out in Iranian patients with cardiac defects. We suggest that 22q11.2 imbalances should be considered in patients with cardiac lesions to provide an accurate diagnosis and appropriate genetic counseling in affected families.



[ DOI: 10.18502/ijml.v9i1.9087 ]

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## Introduction

Congenital heart defects (CHD) with an 0.85-1% incidence of live births are the most common congenital disabilities [1]. The genetic etiology of CHDs includes both single-gene mutations and chromosome abnormalities, about 10% and 9-18% of CHD, respectively [2, 3]. Different studies have detected an increased number of rare copy number variations (CNVs), contributing to the risk of CHDs [4-7]. CNVs contribute to 10-15% of CHD based on clinical and research findings [8]. It is expected that these CNVs affect one or more dosage-sensitive genes implicated in heart development. Among these, 22q11.2, 8p23.1, and 5q35.1 deletions and/or duplications containing *TBX1*, *GATA4*, and *NKX2-5* cardiac-related genes, respectively, are remarkable [4, 9, 10].

The majority of CHDs are isolated, which means cardiovascular malformation is the only major phenotype in the patient at the time of diagnosis. About 25% of CHDs are associated with extracardiac anomalies and/or might be a part of a genetic syndrome [11]. Although many genomic syndromes are strongly associated with CHDs like Di-George, Williams–Beuren, Wolf-Hirschhorn, and Cri-du Chat syndromes, CNVs have also been reported in isolated CHDs [7, 12]. Multiplex Ligation-dependent Probe Amplification (MLPA) is a targeted technique for detecting known CNVs. The MLPA technique is a cost-effective, reliable, and rapid test with high specificity and sensitivity [13]. This study has been performed using MLPA kit P311-B1 to detect deletions/duplications of five critical heart developmental genes, including *TBX5*, *GATA4*, *BMP4*, *NKX2-5*, and *CRELD1* (3p25) and also the chromosomal region of 22q11.2, in 70 Iranian patients with familial and sporadic non-syndromic CHDs.

## Materials and Methods

### Clinical information

Seventy Iranian patients with various ethnic origins were included in the current study. All patients were referred to the pediatric cardiology and neonatal intensive care unit (NICU) of Tehran Children Medical Center, Tehran University of Medical Sciences, from February 2016 to May 2018. The patients' median age at the study time was 18 months (2 months - 15 years).

Twenty patients out of 70 were familial, and the other 50 were sporadic. In familial cases, there were at least two affected patients (first- or second-degree relatives) with non-syndromic CHD regardless of the CHD type (albeit the same type of CHD in each family was preferred). The type of CHD in sporadic cases was not important. Congenital cardiac malformations were diagnosed by echocardiography. Patients showed different kinds of heart anomalies (Tables 1,2). All patients were carefully assessed for any extracardiac features and for possible maternal exposure to teratogenic factors. Moreover, familial cases with a history of recurrent abortions were examined for balanced chromosomal abnormalities using the GTG-banding technique.

**Cytogenetic analysis**

Classical cytogenetics was performed according to standard protocol. High-resolution GTG



[ DOI: 10.18502/ijml.v9i1.9087 ]

bandings (480-520 resolution) were used to exclude chromosomal abnormality in the examined patients.

**Molecular analysis**

Genomic DNA was extracted using the standard salting-out method. SALSA MLPA Kit P311-B1 (MRC-Holland, Amsterdam, The Netherlands) was used in our study, which has been designed for CNV screening in 5 important cardiac-related genes, including *TBX5* (12q24), *GATA4* (8p23), *BMP4* (14q22), *NKX2*-5 (5q35) and *CRELD1* (3p25) and also the chromosomal region of 22q11.2. We followed the manufacturer's protocol as described in the MRC-Holland catalog. MLPA results were analyzed using Gene Marker (Softgenetics LLC, State College, Pa., USA) and Coffalyser.Net software (MLPA analysis tool developed by MRC-Holland). The MLPA test was repeated for each observed variation to confirm the result. The test was also executed for parents of patients with abnormal results to determine whether it is *de novo* or inherited. This research study was approved by the Ethics Committee of the University of Social Welfare and Rehabilitation Sciences of Tehran, Iran (IR.USWR.REC.1395.132).

**Table 1.** Clinical data of familial cases

| Family No. | CHD phenotype | Inheritance pattern | Family No. | CHD phenotype | Inheritance pattern |
|---|---|---|---|---|---|
| **1** | Single ventricle (Small left ventricle), Large VSD | AD | 11 | Large PM/VSD, Small PFO/ASD, AVSD, PH | AR/AD |
| **2** | Large PDA, PFO | AD | 12 | TOF | AD/AR |
| **3** | Large VSD (Sub Aortic), sever Co A | AD | 13 | Large Apical VSD, PS | AD |
| **4** | Secundum ASD | AR/AD | 14 | Large ASD/ Functionaly single Ventricle (left ventricle) | AR/AD |
| **5** | PA/VSD, MAPCA | AR | 15 | Small ASD, large VSD | AD |
| **6** | Sever ASD | AD | 16 | Large PM/VSD, PS (sub valvar and valvar) | AD |
| **7** | TOF | AD | 17 | BAV, mild AS, mild AR, mild AS | AD |
| **8** | Secundum ASD | AR/AD | 18 | ASD | AD |
| **9** | TOF | AD | 19 | VSD | AD |
| **10** | BAV | AR | 20 | VSD/PS | AD |

VSD= Ventricular septal defects; AD= Autosomal recessive; AR= Autosomal dominant; PFO= Patent foramen ovale; ASD= Atrial septal defect; AVSD= Atrioventricular septal defects; PDA= Patent ductus arteriosus; COA= Coarctation of the aorta; PA= Pulmonary atresia; MAPCA= Multiple aortopulmonary collateral arteries; TOF= Tetralogy of fallot; BAV= Bicuspid aortic valve; PS= Pulmonary stenosis



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**Table 2.** Number of defects detected by echocardiography in sporadic cases

| Echo | Numbers | Echo | Numbers |
|---|---|---|---|
| **Atrial septal defect** | 7 | Coarctation of the aorta/Bicuspid aortic valve | 1 |
| **Patent ductus arteriosus** | 4 | Large ventricular septal defects / sever pulmonary stenosis | 1 |
| **Ventricular septal defect** | 4 | Alcapa | 1 |
| **Tetralogy of fallot** | 5 | Left ventricular outflow tract obstruction | 2 |
| **Total anomalous pulmonary Venous connection** | 2 | Large discrete semicircular subaortic stenosis | 1 |
| **Pulmonary stenosis** | 3 | Side by side ventricle- Ventricular septal defect – Pulmonary hypertension | 1 |
| **Pulmonary atresia/ Ventricular Septal defect/ Multiple aortopulmonary collateral arteries** | 1 | Pulmonary valve stenosis/ Tricuspid regurgitation/ patent foramen ovale | 1 |
| **Patent ductus arteriosus/ Atrial septal defect** | 1 | Moderate left atrioventricular valve regurgitation – Complete atrioventricular septal defect | 1 |
| **Aortic valve/ Ventricular septal defect** | 1 | atrial septal defect / Ventricular septal defect/ tricuspid atresia | 1 |
| **Double outlet right ventricle/ Sever pulmonary stenosis/ Ventricular septal defect** | 1 | Large ventricular septal defect/ large patent ductus arteriosus/ bicuspid aortic valve/ sever, Pulmonary hypertension | 1 |
| **Double outlet right ventricle/ Mild pulmonary stenosis/ Trivial aortic insufficiency** | 1 | Severe Coarctation of the aorta/ Ventricular septal defects/ Patent ductus arteriosus | 1 |
| **Ventricular septal defect/ Pulmonary atresia** | 1 | others | 7 |

## Results

### Cytogenetic analysis

None of the patients and their parents (with a history of two or more spontaneous pregnancy losses) showed chromosome abnormality.

### Molecular analysis

No CNV was observed in *GATA4*, *BMP4*, *NKX2*-5, *TBX5*, or *CRELD1* genes in all the familial or sporadic patients (70 cases). However, in 2 out of 50 sporadic patients, the MLPA test identified a heterozygous deletion in the 22q11.2 region, including *CDC45*, *GP1BB*, and *DGCR8* genes. One of the patients was diagnosed with Tetralogy of Fallot (TOF) and the other one presented with Ventricular Septal Defect (VSD)/ Pulmonary atresia (PA)/ major aortopulmonary collateral arteries (MAPCAs) that is also considered as a form of TOF. Moreover, we detected a duplication of 22q11.2, including all the *CDC45*, *GP1BB*, and *DGCR8* genes in the third patient with Patent Ductus Arteriosus (PDA). All three patients' parents showed typical results for the 22q11.2 region, indicating a de novo basis for the CNVs in this region.

## Discussion

According to different studies, rare CNVs contribute to all forms of CHD. CNVs' involvement in CHD etiology has been





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reported in both syndromic CHD and isolated CHD [14, 15]. Although, as expected, the diagnostic yield of CNVs is higher in syndromic CHD [18]. In a meta-analysis of array CGH studies in prenatally diagnosed CHD, Jansen et al. showed a diagnostic yield of 12% for detecting CNVs in the unselected population of fetuses with CHD. In this report, the CNV detection rate was about 3.4% when only isolated CHDs were considered; however, the latter was made when 22q microdeletions had been excluded [19]. Postnatal studies also support an excellent yield of detecting pathogenic CNVs in isolated and syndromic CHD [18, 20]. In a study performed by Geng et al., the diagnostic yield of CNV detection was 14.1-20.6% for syndromic CHD and 4.3-9.3% for isolated CHD [18].

In the present study, we used MLPA to survey the 22q11.2 region and *GATA4*, *NKX2-5*, *TBX5*, *BMP4*, and *CRELD1* genes for CNV in 70 patients with isolated CHD (50 sporadic and 20 familial cases). No CNV was detected in familial patients. It was not surprising since our studied group was small, and more importantly, the monogenic mutations are the most common etiology in pedigrees with familial recurrence of CHD [2, 21]. Accordingly, Nozari et al. performed the whole exome sequencing technique for these familial cases and identified monogenic variants in some patients [22]. Of 50 patients with sporadic isolated CHD, three cases were identified with 22q11.2 copy number variation (6%); two of them showed deletions (4%), and one had duplication (2%). In two other similar studies which had used MLPA for CNV analysis in CHD, Li et al. reported an identification rate of about 4% for 22q11.2 CNVs (3% for deletion and 0.6% for duplication of the same region) [9], and Mutlu et al. showed a diagnostic yield of 6.7% for 22q11.2 CNVs, all of which were deletions. The patients' population in the first group included both syndromic and isolated CHD, but the latter had studied only non-syndromic patients. None of these studies identified CNV in genes present in the MLPA kit p-311 [23].

Several readings mention that one of the most common CNVs contributing to CHDs is 22q11.2 deletion [24]. 22q11.2 deletion syndrome, affecting approximately 1 in 4,000 individuals, is the most common genetic syndrome. CHD is present in 60%-75% of patients with 22q11.2 microdeletion in different forms as well as interrupted aortic arch type B, truncus arteriosus, TOF, and VSD in about 50%, 33%, 15%, and 5–10% of cases, respectively [24, 25-28]. The 22q11DiGeorge syndrome phenotype is highly variable, including short stature, hypocalcemia, immunodeficiency, dysmorphic facial features, palatal anomalies, cognitive impairment, various neuropsychiatric disorders, and cardiac defects [25].

In our study, the detection rate of 22q11.2 deletion was 2 of 50 (4%). One of the patients presenting with 22q11DS had TOF, and the other one showed VSD/ PA/ MAPCAs that is also considered as a form of TOF. TOF is the most common cyanotic congenital cardiac disease, with 1 per 3000 live births. TOF accounts for 10% of all CHDs [29-30]. In two different studies performed by Greenway et al.



[ DOI: 10.18502/ijml.v9i1.9087 ]

and Gioli-Pereira et al. on sporadic isolated TOF, 22q11.2 deletions were identified in 2 of 114 (~2%) and 8 of 123 (6.5%) patients, respectively [7, 31]. The latter group suggested non-syndromic TOF patients with 22q11 microdeletion have a higher risk for pulmonary atresia. Genetic screening would be necessary for the correct supervision and more specific genetic counseling.

22q11.2 duplication is the reciprocal product of the same region deleted in 22q11.2DS, and its frequency is about one-half of that of deletion 22q11.2 [32]. Patients with this duplication perform with a highly variable phenotype ranging from normal to severely affected. The clinical characteristics include facial features, congenital heart defects, learning disabilities, hearing loss, and development delay, some of which are similar to the 22q11.2DS phenotype. This phenotypic variability for 22q11.2 microduplication syndrome can explain why these patients are diagnosed less frequently than its corresponding microdeletion syndrome [33, 34]. According to Hasten et al., the prevalence of CHD in 22q11.2 duplication is 25%, and cardiac anomalies occur in the broader spectrum compared to 22q11.2 deletion [35]. We detected 1 of 50 patients (2%) with 22q11.2 duplication in the present study. The heart defect in the patient was PDA. Breckpot et al. investigate a cohort of 46 sporadic patients with severe non-syndromic CHD and demonstrated pathogenic CNVs in 2 subjects, one of which was a 22q11.2 duplication which was similar to our study. This group had carried out a careful clinical examination to exclude syndromic cases. The cardiac anomaly in a patient with 22q11.2 duplication was AVSD [17]. In the study performed by Li et al., an identification rate of 0.6% was reported for 22q11.2 duplication. The patient in this study had been diagnosed with PDA without any extracardiac symptoms [9].

## Conclusion

Large CNVs spanning several genes affect some other important organs, aside from the heart, is expected. However, it is considered that CHD may be the first diagnosable symptom in these patients. Besides, there is a variable phenotypic expression for some CNVs like 22q11.2 deletions and duplications, and there are several reports for isolated CHDs resulting from CNVs overlapping syndromic regions, including the 22q11.2 CNVs. Therefore, the analysis of CNVs containing cardiac genes in CHD patients, even those with isolated CHD, can lead to an early diagnosis and improved management of the disorder in these patients. More specific genetic counseling can be carried out for such families. This is notably important when patients are infants or young children. Our study suggests that 22q11.2 CNVs have a notable frequency in isolated CHD and should not be considered only for syndromic type. MLPA test as a relatively fast and inexpensive technique can be utilized in diagnostic laboratories to identify these CNVs despite the presence or absence of extracardiac anomalies.

## Conflict of Interest

The authors declare that they have no competing interests.





[ DOI: 10.18502/ijml.v9i1.9087 ]

## Acknowledgments

We greatly appreciate the University of Social Welfare and Rehabilitation Sciences research deputy for funding this project (Grant Number: 801/95/5/1249). We would also like to thank the patients and their families for contributing to this study. We appreciate the help of the laboratory staff of the Genetics Research Center of the University of Social Welfare and Rehabilitation Sciences. We also thank the Children Medical Center of Tehran University of Medical Sciences staff for their excellent assistance in recruiting and providing clinical data.

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[ DOI: 10.18502/ijml.v9i1.9087 ]