



Molecular characterization of patients with clinical suspicion of 22q11.2 deletion syndrome

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Background

22q11.2 deletion syndrome (22q11.2 DS) is one of the most common genetic causes of learning disability. The aim of the study was to characterize genetically the individuals with clinical suspicion of 22q11.2 DS using sequential cytogenetic approach.

Patients and methods

The present study was conducted on 20 patients with features suggestive of 22q11.2 DS including cardiac, velopharyngeal, craniofacial, and other dysmorphic features. Cytogenetic and molecular analyses were performed by conventional karyotyping and fluorescence in-situ hybridization (FISH). Multiplex ligation-dependent probe amplification analysis (MLPA) was done for cases clinically suspected of being affected with 22q11.2 DS and showing no deletion by FISH.

Results

Conventional karyotyping revealed two (10%) cases and FISH analysis revealed four cases with deletion in the 22q11.2 region. No more cases were detected by MLPA analysis compared with FISH.

Conclusion

The diagnosis of 22q11.2 DS is primarily suspected by clinical picture. The cytogenetic diagnosis can be greatly improved by using FISH analysis, which detects the typical deletion in the 22q11.2 low copy repeats A–B, whereas atypical or distal deletion in 22q11.2 low copy repeats B–C, B–D, or C–D can be detected by MLPA analysis, which will allow more precise determination of the size and location of the deletion.

Keywords:

22q11.2 deletion syndrome, conventional cytogenetic analysis, fluorescence in-situ hybridization, multiplex ligation-dependent probe amplification

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Introduction

22q11.2 deletion syndrome (22q11 DS) is the most common recurrent microdeletion in humans and is one of the most common genetic causes of learning disabilities and mild mental retardation. It has an incidence of 1 per 4000 live births (Scambler, 2000; McDonald-McGinnandSullivan, 2011).

Individuals with 22q11.2 DS have highly variable phenotypes with multisystem involvement, mainly including congenital heart disease (CHD), velopharyngeal anomalies, hypoparathyroidism, T-cell immunodeficiency, craniofacial features, cognitive deficits, and high rates of psychiatric morbidity (Green *et al.*, 2009; Mitchell, 2015).

It has been now recognized that 22q11.2 DS includes the phenotypes described as DiGeorge syndrome (DGS), Shprintzen or velocardiofacial syndrome (VCFS), conotruncal anomaly face syndrome (Takao syndrome), some cases of

autosomal-dominant Opitz G/BBB syndrome, and Cayler cardiofacial syndrome (asymmetric crying facies), in addition to isolated outflow tract defects of the heart including tetralogy of Fallot, truncus arteriosus, and interrupted aortic arch (McDonald-McGinnandSullivan, 2011).

DGS was originally described as a developmental field defect of the third and fourth pharyngeal pouches and was identified in neonates, with hypoplasia of the thymus and parathyroid glands. Later, CHD, specifically conotruncal cardiac anomalies, was added. Most of the individuals with DGS were identified in the neonatal period with a major congenital heart defect, hypocalcemia, and immunodeficiency (Scambler, 2000).

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VCFS was originally described as the combination of palatal anomalies, velopharyngeal incompetence, CHD (usually a ventricular septal defect or tetralogy of Fallot), characteristic facial features, and developmental delay or learning difficulties. Children with VCFS were mostly diagnosed in cleft palate clinics or craniofacial centers when they reached school age and speech and learning difficulties became evident. VCFS may also be referred to as Shprintzen syndrome (Green *et al.*, 2009).

In conotruncal anomaly face syndrome or Takao syndrome, the cardiovascular presentation is the focus of attention. Takao syndrome may be more appropriate for those cases with a preponderant cardiac presentation in contrast to the low T-cell and hypocalcemic presentation in infancy of DGS and the craniofacial and palatal abnormalities typical of Shprintzen syndrome. These three phenotypes may be seen in the same family, and most cases of all three categories have been shown to have a 22q11 deletion (McDonald-McGinnandSullivan, 2011; Mitchell, 2015).

Chromosome 22q11.21 region contains a cluster of low copy repeats (LCRs) of high-homology sequences, labeled A–H (LCRs A–H). These LCRs can mediate nonallelic homologous meiotic recombination leading to recurrent copy number variations with a resulting deletion or duplication of variable segments within this chromosomal region (Shaikh *et al.*, 2000; Stankiewicz and Lupski, 2002).

Approximately 97% of patients with 22q11.2 DS have a common recurrent deletion of ~3 Mb (known as DiGeorge critical region) in one copy of chromosome 22. It is flanked by LCRs A–D or less commonly, a shorter 1.5 Mb nested deletion (LCR A–B) (Yagi *et al.*, 2003; Ben-Shachar *et al.*, 2008). Both are proximal deletions that usually lead to similar phenotypes.

More distal deletions were classified to type 1 (flanked by LCRs C–E, D–E, and D–F), type 2 for deletions flanked by LCR E–F, and type 3 for deletions minimally spanning the LCR F–G and encompassing the SMARCB1 gene (601607). All the three distal deletions are pathogenic with a milder phenotype that lacks growth restriction and cardiovascular defects (Shaikh *et al.*, 2000; Mikhail *et al.*, 2014).

Nested regions of the 3 Mb DiGeorge critical region interval and central deletions have a high rate of inheritance, whereas the loss of the entire region and distal deletions are usually *de novo* (Burnside, 2015).

The most important candidate gene in 22q11.2 DS is TBX1 (602054). Haploinsufficiency of this gene

was described to be particularly responsible for five major phenotypes of the 22q11.2 syndrome, namely, abnormal facies, cardiac defects, thymic hypoplasia, velopharyngeal insufficiency, and parathyroid dysfunction. Nevertheless, it did not appear to be responsible for the typical mental retardation seen with 22q11.2 DS (Yagi *et al.*, 2003).

For central deletions, CRKL is a candidate gene (Racedo *et al.*, 2015), whereas MAPK1/ERK2 genes are considered candidates for the distal type 1 deletions (Newbern *et al.*, 2008; Samuels *et al.*, 2008). SMARCB1 is the critical gene specifying distal type 3 deletions with high rate of malignant rhabdoid tumors (Ben-Shachar *et al.*, 2008).

Owing to highly variable and overlapping features of patients with 22q copy number changes, the genotype–phenotype correlations are extremely difficult to be predicted accurately. In some patients, symptoms may not rise to a severity level that would meet thresholds for diagnostic policy.

The common diagnostic procedure used for detection of deletions and duplications at 22q11.2 is conventional chromosomal analysis followed by fluorescence in-situ hybridization (FISH). Standard karyotype analysis will only detect 10–20% of 22q11 deletions. FISH analysis usually relies on using a single fluorescent commercial probe [N25 or TUP-like enhancer of split gene 1 (TUPLE1)], located proximally in the typically deleted region. Thus, it fails to detect deletions that are either proximal or distal to the FISH probes and does not provide any information about the length of the deletion (Rump *et al.*, 2014; Burnside, 2015). In recent years, new approaches for better characterization of 22q11.2 deletions have been developed including array comparative genomic hybridization, multiplex ligation-dependent probe amplification (MLPA), and multiplex quantitative real-time PCR (Jalali *et al.*, 2008; Bittel *et al.*, 2009).

The aim of the study was to characterize genetically the individuals with clinical suspicion of 22q11.2 DS. Sequential cytogenetic approach including karyotypic analysis, FISH, and MLPA was applied for proper molecular characterization and clinical correlation.

Patients and methods

The present study was conducted on 20 patients with features suggestive of 22q11.2 DS including cardiac, velopharyngeal, craniofacial, and other dysmorphic features. The patients were referred to the Cardiology Clinic, Abo El-Reech Hospital, Kasr El Ainy Medical School, and the Clinical Genetics Department,



National Research Center. Informed consents were obtained from parents of studied participants. The study design was approved by the Scientific Research Committee of the Clinical Pathology Department, Faculty of Medicine, Cairo University, and by the Medical Research Ethics Committee of National Research Center. Data confidentiality was observed according to the Revised Helsinki Declaration of Bioethics.

The studied patients included 11 males and nine females, with ages ranging from 2 months to 17 years. All patients were subjected to medical history including parent's occupation, pregnancy and delivery histories, family history, and history of developmental milestones. A detailed history of cardiac symptoms such as dyspnea, palpitation, arrhythmia, syncope was stressed upon. History of abnormal feeding, recurrent infections, convulsions, behavioral disorders, or other symptoms related to the syndrome was also included. Pedigree analysis included three generations, taking into consideration consanguinity, similarly affected siblings, and other abnormalities in family members.

Detailed clinical examination, with special emphasis on heart, craniofacial dysmorphic features, ear, nose, and throat examination in addition to the proper assessment of different body systems including the central nervous system, was performed. Anthropometric measurements including height, length, weight, and head circumference were assessed. They were compared with age-matched and sex-matched healthy controls that were enrolled from Surgery Clinic, Abo El-Reesh Hospital, Kasr El Einy Medical School.

Chest radiography and ECG were carried out whenever indicated.

Cytogenetic studies and fluorescence in-situ hybridization analysis

Chromosomal analysis was performed from peripheral blood samples using GTG-banding technique (Seabright, 1971). Karyotype description followed the International System for Human Cytogenetic Nomenclature recommendations (ISCN, 2016).

FISH was carried out with commercial probes according to the manufacturer's instructions, using DiGeorge/VCFS TUPLE1/TBX1 or N25 and 22q13.3 DS probe combinations. They are specific for genes lying within the minimal DGCR (including LCRs A–B), combined with a control probe for 22q13.3 deletion, and they are labeled red and green, respectively (Cytocell Inc., Cambridge, UK). At least 100 interphases were scanned in each patient

using Zeiss imaging system with color fluorescence filters. Only metaphases and interphases with clear, nonoverlapping signals were analyzed.

Multiplex ligation-dependent probe amplification assay

Genomic DNA extraction from 5 ml peripheral blood lymphocytes was carried out using PAXgene tube and PAXgene Blood DNA kit catalog no./ID: 761133 (PreAnalytix, Hiden, Germany). **MLPA assay was performed using the SALSA MLPA probemix P250-B2 DiGeorgekit (MRC-Holland, Amsterdam).** This probemix is designed to detect deletions or duplications in the human 22q11.2 region including LCRs A–H. It contains 48 probes with amplification products between 129 and 487 nucleotides: 29 probes are located in the 22q11.2 region to detect most common types of deletion, in addition to 22q11.1 region for cat eye syndrome. Nineteen probes are present for relevant regions of DGS type II or disorders with phenotypic features overlapping with DGS on 22q13 and on chromosomes 4q, 8p, 9q, 10p, and 17p. These 19 probes are also used as reference probes.

Three normal age-matched males and females were included as an internal quality control. An amount of 100–200 ng DNA in a final volume of 5 μ l was used for the assay which was run according to the manufacturer's instructions. **The results were analyzed with the Coffalyser software that has integrated functions specific for the analysis of data derived from the MLPA reactions.**

When a target locus in a test sample is disomic (i.e. present in a normal copy number; $n = 2$), the normalized signal value for that locus will be equal to that of the control sample, and thus, the dosage quotient will be close to 1.0. A deletion of a locus in a test sample should result in a reduction of relative signal strength of greater than 30%, whereas a duplication should lead to an increase in relative signal strength of greater than 30%. Thus, in the case of a deletion or duplication, the normalized signal should be less than 0.7 ($1 - 0.3$) or greater than 1.3 ($1 + 0.3$), respectively. A δ -value of 0.3 is a commonly accepted empirically derived threshold value for genetic dosage quotient analysis (Galli *et al.*, 2003).

Results

The present study was conducted on 20 patients with features suggestive of 22q11.2 DS. The patients were referred to the Cardiology Clinic, Abo El-Reesh Hospital, Kasr El Ainy Medical School, and the Clinical Genetics Department, Medical Centre for

Scientific Excellence, National Research Center. There were 11 male and nine female patients, with a female to male ratio of 1: 1.2; their ages ranged from 2 months to 17 years, with a mean value of 3.7 years. Parental consanguinity was evident in 45%, and 15% had a family member with similar features, whereas one case had a sibling who died of cardiac arrest.

Patients showed many somatic abnormalities including cardiac, velopharyngeal, craniofacial, and other dysmorphic features with or without developmental delay or neurological abnormalities (clinical features were presented in Table 1, Fig. 1).

Two (patients 17 and 20) (10%) patients showed DGS deletion by conventional cytogenetic analysis. Eight (45%) patients had normal 46, XX female karyotype and 10 (55%) patients had normal 46, XY male karyotype.

Regarding FISH analysis, 16 (80%) patients showed normal FISH results, whereas four (20%) patients showed DGS deletion in all studied metaphases and interphase nuclei (patients 10, 17, 19, and 20) (Fig. 2).

MLPA analysis was conducted on 16 individuals clinically suspected of being affected by 22q11 DS and showed normal FISH results, in addition to a patient with DGS deletion (patient 17), as a positive control, which revealed the typical deletion extending between LCRs A-D. All other patients examined by MLPA showed negative results (Fig. 3).

Table 1 Summary of clinical data of patients showing normal fluorescence in-situ hybridization results (n=16)

Features	n (%)
Skull abnormalities	
Microcephaly	10/16 (62.5)
Brachycephaly	2/16 (12.5)
High anterior hairline	2/16 (12.5)
Dysmorphic features	13/16 (81.25)
Long philtrum	7/16 (43.75)
Cleft palate	3/16 (18.75)
Epicanthal folds	6/16 (37.5)
Sparse eyebrows	3/16 (18.75)
Low-set ears	5/16 (31.25)
Abnormalities of nose	11/16 (68.75)
Anomalies of upper and lower extremities	13/16 (81.25)
Cardiac anomalies	15/16 (93.75)
Tetralogy of fallot	2/16 (12.5)
Ventricular septal defects	2/16 (12.5)
Pulmonary artery stenosis	4/16 (25)
Atrial septal defects	3/16 (18.75)
Atrioventricular canal	3/16 (18.75)
Patent foramen ovale	1/16 (6.25)
Neurological anomalies	3/16 (18.75)
Hypotonia	2/16 (12.5)
Generalized tonic clonic seizures	1/16 (6.25)
Developmental delay	10/16 (62.5)

Discussion

One of the most common genetic disorders causing learning disabilities and mild mental retardation is 22q11.2 DS, with an incidence of one per 4000 live births. Approximately 75% of children with 22q11 DS have CHD, which represents the major cause of mortality in 22q11.2 DS (Yi *et al.*, 2014).

DGS is one of 22q11 microdeletion syndromes. Definitive diagnosis classically depends on combining FISH technique with conventional karyotyping, which is costly and time consuming. Moreover, FISH can only detect deletions that are restricted to the proximal 3 Mb of typical deleted region and does not provide details about the length of the deletion (Rump *et al.*, 2014; Burnside, 2015).

The present study investigated patients with features suggestive of 22q11.2 DS by the classic diagnostic approach, using GTG karyotyping combined with FISH analysis. MLPA assay was applied for patients not diagnosed with the former techniques to study the diagnostic yield of MLPA over FISH analysis to establish the most proper diagnostic procedure for those patients. Rapid and accurate diagnosis is critical for early proper management and genetic counseling.

We revealed that of all patients, two (10%) patients with DGS showed deletion in the 22q11.2 region. By FISH analysis, we found four DGS cases (20%) exhibited the deletion. On the contrary, 16 (80%) patients had no deletion in metaphase or in interphase nuclei. Similar results were found by the study done by McDonald-McGinn *et al.* (2010). On the contrary, Michaelovsky *et al.* (2012) detected 22q11.2 deletion in 77.8% (109/140) of their studied patients using FISH analysis. This high difference may be due to different sample size and the selection criteria of the patients in the latter, which included previously diagnosed and referred cases.

The majority (90%) of the 22q11.2 deletions are of 3 Mb and often referred to as typically deleted region, whereas 8% are 1.5 Mb in size (~28 genes) (LCR A-B). Some rare atypical deletions of shorter size and in variable locations have also been reported (Beaujard *et al.*, 2009). FISH technique which is still in routine use in many laboratories is unable to detect deletions that are either proximal or distal to the particular FISH probe used necessitating further investigations to diagnose negative cases by FISH (Rump *et al.*, 2014).

In recent years, several new methods for detecting 22q11.2 deletion have been developed: comparative genomic hybridization (Urban *et al.*, 2006), MLPA (Jalali *et al.*, 2008), multiplex quantitative

Figure 1

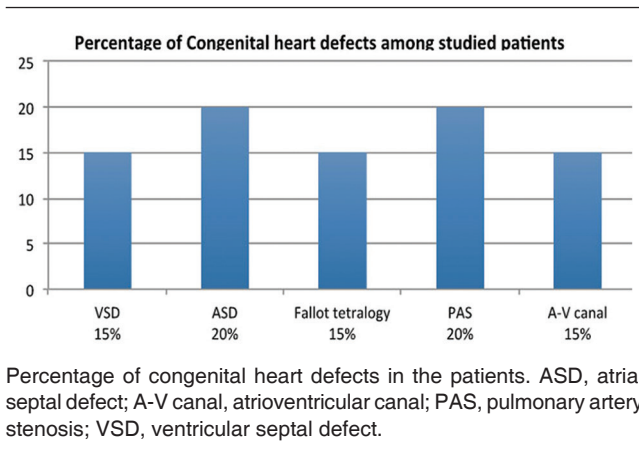


Figure 2

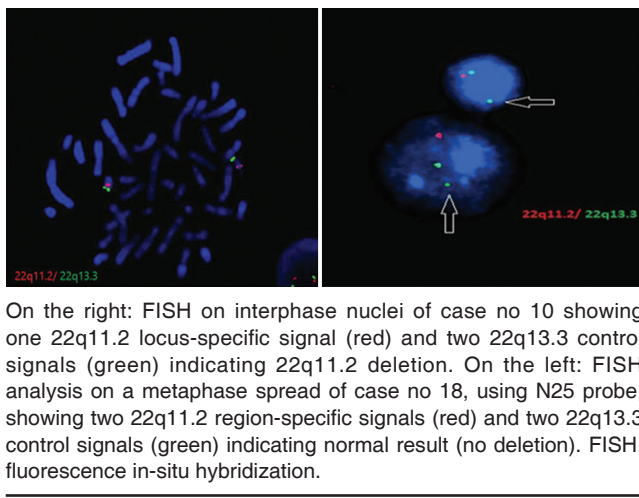
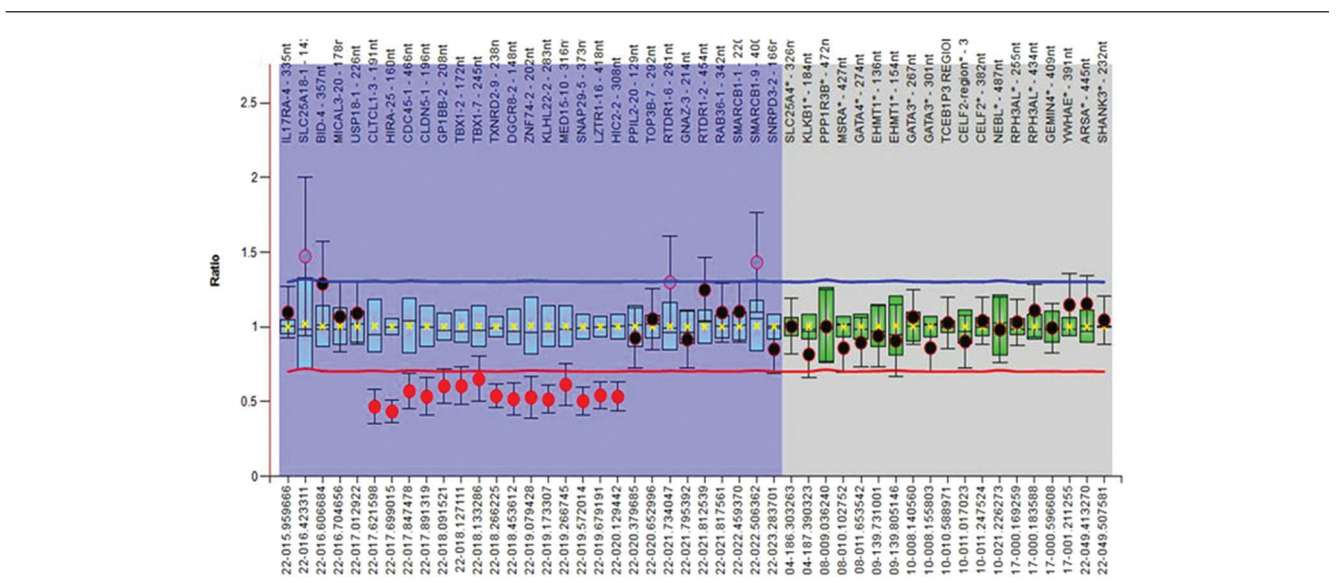


Figure 3



real-time PCR (Tomita-Mitchell *et al.*, 2010), and high-resolution SNP microarray analysis (Busse *et al.*, 2011). The aforementioned whole-genome methods are still at the research stage and require expensive equipment for performing the assay and analyzing the data. MLPA is a rapid and easy economical method for the simultaneous diagnosis of multiple anomalies with a simple kit.

In our work, MLPA analysis was performed in those 16 individuals clinically suspected of being affected with 22q11.2 DS while showing normal results on both karyotyping and FISH analyses. It was also done for a DGS case with 22q11 deletion detected by FISH as a confirmation for MLPA technique. We did not detect 22q11.2 deletion among the patients. Other studies revealed similar results by FISH and MLPA (Michaelovsky *et al.*, 2012; Pires *et al.*, 2014).

All cases showing deletion by FISH in the present study have abnormal facies and velopharyngeal insufficiency, whereas cases no 10, 17, and 19 have cardiac defects. TBX1 genes deletions are probably the cause of these phenotypes according to Yagi *et al.* (2003) and Gao *et al.* (2015).

Jerome and Papaioannou (2001) investigated the potential role of the TBX1 gene in DGS/VCFS by producing a null mutation in mice. They found that heterozygous mice for the mutation had a high incidence of cardiac outflow tract anomalies whereas homozygous mice displayed a wide range of developmental anomalies

The chart is showing deletion at the 22q11.2 LCR (A-D) region for case no 17. The deletion is denoted by the red spot below the deletion cut-off line (red) in the ratio chart. Longitudinal axis represents the final ratio after comparing the patient and control sample peak intensities by a dosage ratio and transverse axis represents the genes covered by the SALSA MLPA probemix, P250-B2DiGeorge kit. The red line represents the 0.7 ratios, and the blue line represents the 1.3 ratios. MLPA, multiplex ligation-dependent probe amplification analysis; LCR, low copy repeats.

encompassing almost all of the common DGS/VCFS features. This hypothesis was later supported by the study of Theveniau-Ruissy *et al.* (2008).

According to the study by Farrell *et al.* (1999) on neural crest-derived tissues in both chick and mouse embryos, functional attenuation of HIRA (Histone Cell Cycle Regulation Defective Homolog A) gene in the chick cardiac neural crest results in a significantly increased incidence of persistent truncus arteriosus, a phenotypic change characteristic of DGS. Moreover, according to the study by Michaelovsky *et al.* (2012), HIRA gene was one of the genes responsible for the cardiac malformations in 22q11.2 DS. In the present study, the four cases showed HIRA gene deletion by FISH, denoting that HIRA gene deletion may be involved in the cardiac malformation found in these patients (Michaelovsky *et al.*, 2012).

Ear anomalies were present in the four cases showing 22q11.2 deletion by FISH analysis. According to Fuchs *et al.* (2015), haploinsufficiency of TBX1 gene may be responsible for ear disorders in patients with VCFS/DGS.

Cases no 17 and 19 had anal atresia, and the latter had associated hypospadias. Previous investigations reported anal anomalies in cases of VCFS and confirmed deletions of the 22q11 region by FISH. They emphasized the importance to consider VCFS in the differential diagnosis of children with anal anomalies (Nagasaki *et al.*, 2011).

The genital manifestations found in these cases (17 and 19) may be due to the overlapping features between 22q11.2 DS, CHARGE (coloboma, heart defects, atresia of choanae, retardation of growth and development, genital hypoplasia, and ear abnormalities) syndrome, and Opitz G/BBB syndrome. Some researchers consider Opitz G/BBB syndrome to be a type of 22q11.2 DS (Meroni, 2011).

Eleven (68.75%) cases suspected as 22q11.2 DS with normal FISH results had abnormalities in the ears; this finding may be of clinical significance as oculo auriculo vertebral (Goldenhar) syndrome (OAVS), including ear anomalies, vertebral defects, heart disease, and renal anomalies. It is often a sporadic condition or in association with 22q11.2 DS (Stanojević *et al.*, 2000; Beleza-Meireles *et al.*, 2015).

Congenital heart defects were found in 15 (93.75%) cases from the suspected 22q11.2 cases with normal FISH results. Syndromes such as OAVS and Alagille syndrome should be suspected. They include CHD (Lee *et al.*, 2015).

Some recognizable syndromes overlap in many features with 22q11 DS and are considered in the differential diagnosis, which can offer explanation to some of the patients with undetectable deletion by MLPA. These syndromes include CHARGE syndrome, Smith-Lemli-Opitz, Alagille syndrome, VATER association, and OAVS.

Finally, TBX1 gene mutation may occur without 22q11.2 deletion and result in the same clinical phenotype

We can conclude that the diagnosis of 22q11.2 DS is primarily suspected by clinical picture. Conventional GTG banded cytogenetic analysis can diagnose 22q11.2 DS with large deletion. The cytogenetic diagnosis can be greatly improved by using FISH analysis which detects typical deletion in the 22q11.2 LCR (A–B), whereas atypical or distal deletion in 22q11.2 LCR B–C, B–D, or C–D can be detected by MLPA analysis (Evers *et al.*, 2016). MLPA method using multiple probes achieves a good resolution combined with the practicality and affordability of a commercial kit, which is rapidly and easily performed in any experienced laboratory.

If the 22q11.2 microdeletion is suspected based on the clinical features, a targeted technique (e.g. FISH and MLPA) can be employed for rapid diagnosis; however, both chromosomal microarray and MLPA have a higher sensitivity for detection of atypical deletions and will allow more precise determination of the size and location of the deletion (McDonald-McGinn *et al.*, 2010).

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Conflicts of interest

There are no conflicts of interest.

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