22q11.2 Deletion Syndrome: Laboratory Diagnosis and TBX1 and FGF8 Mutation Screening

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Abstract

Velocardiofacial syndrome is one of the recognized forms of chromosome 22q11.2 deletion syndrome (22q11.2 DS) and has an incidence of 1 of 4,000 to 1 of 6,000 births. Nevertheless, the 22q11 deletion is not found in several patients with a 22q11.2 DS phenotype. In this situation, other chromosomal aberrations and/or mutations in the T-box 1 transcription factor C (TBX1) gene have been detected in some patients. A similar phenotype to that of the 22q11.2 DS has been reported in animal models with mutations in fibroblast growth factor 8 (Fgf8) gene. To date, FGF8 mutations have not been investigated in humans. We tested a strategy to perform laboratory testing to reduce costs in the investigation of patients presenting with the 22q11.2 DS phenotype. A total of 109 individuals with clinical suspicion were investigated using GTG-banding karyotype, fluorescence in situ hybridization, and/or multiplex ligation-dependent probe amplification. A conclusive diagnosis was achieved in 33 of 109 (30.2%) cases. In addition, mutations in the coding regions of TBX1 and FGF8 genes were investigated in selected cases where 22q11.2 deletion had been excluded, and no pathogenic mutations were detected in both genes. This study presents a strategy for molecular genetic characterization of patients presenting with the 22q11.2 DS using different laboratory techniques. This strategy could be useful in different countries, according to local resources. Also, to our knowledge, this is the first investigation of FGF8 gene in humans with this clinical suspicion.

Keywords
► velocardiofacial syndrome
► 22q11.2 deletion syndrome
► laboratory diagnosis
► TBX1
► FGF83

Introduction

Velocardiofacial syndrome (VCFS) is one of the recognized forms of chromosome 22q11.2 deletion syndrome (22q11.2 DS) and has an incidence of 1 of 4,000 to 1 of 6,000 live births.1,2 It exhibits an expansive phenotype with more than 180 clinical features involving almost every organ and system. The major features include congenital heart disease, characteristic face, palatal abnormalities, immune deficiency, hypocalcemia, and cognitive or behavioral disorders.1,2 The highly variable phenotype makes it difficult to select patients for genetic testing.1 Different studies have yielded variable results, according to the selection criteria. Recently, a comprehensive study suggested a set of criteria for the 22q11.2 deletion screening.3 The laboratory diagnosis for 22q11.2 deletion has been usually performed by fluorescence in situ hybridization (FISH) using a locus-specific probe.4–6 The multiplex ligation-dependent probe amplification (MLPA) is also a current approach.6 In the past 10 years, the application of chromosomal microarray analyses (CMA) has changed the way we detect genomic imbalances and currently CMA is suggested as a first-tier diagnostic test for individuals with developmental disabilities or congenital anomalies.7 In some countries, the CMA is also a first-tier choice for individuals with a typical phenotype of 22q11.2 DS.1,8 Unfortunately, cost and technical
limitations make it difficult to adopt this technique as a first test-of-choice in several countries, and thus less expensive tests such as FISH or MLPA have been considered an effective alternative for developing countries.9,10

The 22q11.2 region includes the developmental gene T-box 1 transcription factor C (TBX1). Mutations in this gene have been reported in individuals with the VCFS phenotype but no detectable deletion.11,12 TBX1 is a dose-dependent gene, which could be modified by genetics and environmental factors.13,14 Studies with animal models suggested an interaction between Tbx1 and fibroblasts growth factor type 8 (Fgf8) during embryogenesis,15 and also during cardiovascular development.16

The FGF8 gene (mapped to 10q24.32) plays an important role during embryonic development, particularly on cell differentiation.17 In the developing embryo, Fgf8 is expressed in limbs, primitive streak, heart, branchial arch, olfactory epithelium, pharyngeal region, and various regions in the central nervous system.18–20 Mutant mice for Fgf8 display cardiovascular, glandular, and craniofacial malformations similar to those seen in human 22q11.2 DS; suggesting that FGF8 may function as a modifier of this syndrome and may contribute to the phenotypic variability.17 However, FGF8 has not been previously studied in patients without 22q11.2 deletion and VCFS phenotype.

This article discusses screening for 22q11.2 deletion on individuals with clinical suspicion of VCFS, using karyotype, FISH, and MLPA. In addition, we performed sequencing of TBX1 and FGF8 genes on selected individuals without 22q11.2 deletion.

Patients and Methods

This study was approved by the Ethics Committee Board of University of Campinas (number 487/2009) and all patients or their legal guardians agreed to participate in this study, by signing an informed consent form.

Patients

The cohort was composed of 109 patients with clinical suspicion of 22q11.2 DS prospectively selected as part of a multicenter study, the Brazil’s Craniofacial Project (CFBP). Before data collection, a standardized clinical protocol was established by all clinical geneticists, involved in this study. The protocol included physical examination, clinical and image investigation for palatal abnormalities (including data of nasopharyngoscopy), and cardiologic assessment (including echocardiogram). Also, included in the protocol was the collection of each patient’s history of hypocalcemia and immunologic alterations/recurrent infections, growth and development, behavioral and neuropsychiatric disorders, and sensorineural and/or conductive hearing loss; as well as conducting neurologic, ophthalmologic, gastroenterologic, genitourinary, and skeletal evaluation.

All patients were evaluated on-site by the clinical geneticists, who completed the aforementioned clinical protocol. This study included patients with at least two common signs found in VCFS, such as palatal abnormalities, congenital heart disease, and typical facial dysmorphisms.

Laboratory Investigation

G-banding and FISH analyses were performed on metaphase preparations of peripheral blood lymphocytes using standard techniques. Genomic DNA was extracted from peripheral blood following standard protocols. 22q11 deletion screening was performed by FISH, using the TUPLE1 probe (Kreatech Diagnostics, Amsterdam, The Netherlands) and/or by MLPA, using the P250-A1 kit (MRD-Holland, Amsterdam, The Netherlands), following the manufacturer’s instructions. This P250 kit is a “high density” 22q11 probe mix, containing 30 different probes targeting the 22q11 region and can be used to distinguish the most common types of deletion and detect atypical deletions. In addition, this kit includes probes for other chromosomal regions, such as 10p14 (DiGeorge syndrome/velocardiofacial syndrome complex 2), 4q35, and 17p13, where imbalances have been detected in patients with similar features to that of VCFS. MLPA data analysis was made against up to five control samples using an in-house Excel spreadsheet National Genetics Reference Laboratory, Manchester, UK.

The breakpoints of chromosomal abnormalities found in two patients were characterized by array genomic hybridization (aGH) analyses, using the genome-wide human le nucleotide polymorphism (SNP) array 6.0 (Affymetrix, Santa Clara, California, United States) and analyzed with GeneChip Operating Software (Affymetrix). Patients selected for this test are described in the results section and further discussed.

Nondeleted individuals were reevaluated and only individuals who remained with a suspicion of 22q11.2 DS, and with no other diagnostic hypothesis were selected for TBX1 and FGF8 gene sequencing analysis. Primers used for both genes have been previously reported by Simioni et al22 and Freitas,23 respectively. The fragments obtained by polymerase chain reaction were directly sequenced using the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, California, United States) and the capillary electrophoresis system (Applied Biosystems 3130xl Genetic Analyzer) was used for fragment reading. The sequences were analyzed with the Chromas Lite (Technelysium Pty Ltd, Australia) and Gene Runner software (Hastings, New York, United States), and compared with annotated reference sequences taken from the Ensembl Genome Browser database.

Results

The karyotype with G-banding analysis was performed for 101 individuals. For eight patients, samples for chromosome analysis were not available. Only one 22q11.2 deletion was detected by chromosomal analysis. Other chromosomal abnormalities were detected in four individuals. In two patients, a marker chromosome was found; one of them also presented with a 22q11.2 deletion and one had no 22q11 deletion. In the latter, the diagnosis was considered nonconclusive, because the origin of the marker chromosome was not identified. In the other two nondeleted individuals, the following chromosomal abnormalities were detected: one with 46,XY,der(11)ins(11;15)(p13; q24q26) (previously published as a case report),22 and one
with 46,XYder(9)ins(9;15)(q33;q21.1–q22.31). These two cases were further characterized by aGH and the final karyotype was defined, respectively, as follow: 46,XY,der(11)ins(11;15)(p13;q24q26),arr8p23.1(8;129,435–11,934,586)x3,11p13(31,706,16031,755,245)x1, 15q25q26(81,869,248–98,962,477)x3; 46,XYder(9)ins(9;15)(q33;q21.1–q22.31),arr15q21.1q22.31(44,275,184–63,567,267)x3 (►Table 1).

All patients were screened for the 22q11.2 deletion by MLPA and/or FISH, according to sample availability. The 22q11.2 deletion was detected in 31 of 109 individuals. In addition, the MLPA analysis revealed an 8p23 duplication in one case (►Table 2).

DNA sequence analysis of TBX1 and FGF8 coding regions was performed in 31 individuals; yet only single nucleotide polymorphisms were present. A summary of the clinical data of patients, including those with and without 22q11.2 deletion is found in ►Table 3.

### Discussion

The 22q11.2 DS phenotype shows markedly variable phenotypic presentation. More than 180 clinical features, both physical and behavioral, have been described and no single clinical feature occurs in 100% of cases. The highly variable phenotype makes it difficult to select patients for genetic screening. Delayed diagnosis of 22q11.2 DS is a significant health problem that may compromise clinical management and hinder genetic counseling.

For this reason, the strategy used in this study was to investigate all individuals with suspicion of this deletion after standardized protocol and clinical information collected by clinical geneticists. This strategy reinforced the clinical heterogeneity and demonstrated the overlapping of clinical features with different conditions.

A conclusive diagnosis was achieved in 33 of 109 (30.2%) cases. Considering that the highly variable phenotype of 22q11.2 DS hampers the selection of patients for genetic testing, the frequency of positive cases in this work is acceptable and even higher than previously. For example, several studies that also included patients with clinical suspicion of 22q11.2 DS, obtained a conclusive diagnosis of between 4 and 20%.

Therefore, the strategy proposed is a good alternative to improve diagnosis in 22q11.2 DS. It was designed specifically for a genetic service with different but limited laboratory resources and increases the value of performing dysmorphologic evaluation before proceeding with confirmatory testing.

Karyotype is still used as a screening method for chromosomal aberrations in several countries. It is also important to complement investigation with MLPA, FISH, and in some cases CMA. Also, the association of karyotype and MLPA has been reported as an efficient alternative to investigate mental deficiency and multiple congenital abnormalities.

The specific investigation of the 22q11.2 DS using karyotype and FISH/MLPA techniques detected 30.2% of the cases in this study. This high detection rate underscores this approach as a low cost, effective strategy, especially if it is preceded by a dysmorphologic evaluation.

Among chromosomal aberrations, deletions in 4q34, 8p12, 10p15, 10p14–15, 14q2–q23, and 17p13.3 have been detected in individuals with suspicion of VCFS. In 4.6% of the cases herein studied, different chromosomal aberrations were detected by karyotype. This result is similar to that obtained in other studies in which the laboratory detection rate ranged between 1.7 and 3.7%.

Some years ago, screening for 22q11 deletion using FISH was the gold standard for 22q11.2 DS diagnosis. It is important to point out that the TUPLE1 FISH probe does not cover atypical deletions involving TBX1 gene region, which may result in false-negative results. This shows the need for different tools to achieve more complete diagnosis in this condition, when aGH is not chosen as the initial approach.

The negative 22q11.2 DS results in the remaining 78 patients reinforce the wide spectrum of unspecific features present in this condition. Taking into account the cost of genetics testing in public health, the strategy herein proposed reduces the need for array techniques, but it is still an important resource to be utilized. It would be the next step; however, this approach is not available routinely in our laboratory.

In addition to other novel chromosomal aberrations and microdeletions, mutations in TBX1 have been reported in individuals with a 22q11.2 DS phenotype. Mice mutant for the Tbx1 gene presented cardiac abnormalities related to VCFS. In this study, no pathogenic mutations in the coding regions of this gene were detected in individuals with clinical suspicion and lacking the 22q11.2 deletion. This result is similar to the result of Gong et al in 2001, in which no mutations in TBX1 gene were detected in 100 individuals with clinical suspicion of VCFS.

### Table 1

| Karyotype Description | n (%) |
|-----------------------|-------|
| 46, XX/XY             | 96 (95) |
| 47, XX, + mar         | 2 (2)  |
| 46, XY, del(22)(q11q11) | 1 (1)  |
| 46, XYder(9)ins(9;15)(q33;q21.1-q22.31) | 1 (1)  |
| 46, XY, der(11)ins(11;15)(p13;q24q26) | 1 (1)  |

### Table 2

| Results Description | n (%) |
|---------------------|-------|
| Typical 22q11.2 deletion (3 Mb) | 30 (27.5) |
| 22q11.2 deletion (3 Mb) and duplication (~ 2.6 Mb) | 1 (0.9)  |
| 8p23 duplication (without 22q11.2 deletion) | 1 (0.9)  |
| No abnormalities detected | 77 (70.7) |

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On the basis of the animal models, the Fgf8 gene was proposed as a potential modulation factor in the VCFS phenotype. The complete inactivation of the Fgf8 signaling molecule, in the mouse leads to pharyngeal and cardiac defects similar to those seen in VCFS in humans. This study seems to be the first investigation of FGF8 mutations in humans with VCFS phenotype. In all investigated cases with clinical suspicion with neither 22q11.2 deletion nor TBX1 gene mutations detected, FGF8 gene mutations were also not found. Failure to detect human FGF8 mutations could be explained by the low number of patients included in this study, or it is possible to suppose that FGF8 has only a minor or even no involvement in VCFS phenotype in humans.

This study presents a strategy of investigation of 22q11.2 DS using different laboratory techniques. It could be useful in different countries, according to local resources. Also, this is the first investigation of FGF8 gene in humans with this clinical suspicion. Further studies would contribute in elucidating the role of FGF8 gene and its interaction with TBX1 gene in the 22q11.2 DS phenotype.

### Table 3 Clinical features of patients with or without 22q11 deletion

| Clinical features                       | With 22q11 deletion, n/N (%) | Without 22q11 deletion, n/N (%) | Total, n/N (%) |
|----------------------------------------|------------------------------|---------------------------------|----------------|
| Congenital heart disease               | 20/30 (66.6)                 | 30/72 (41.6)                    | 50/102 (49.0)  |
| Conotruncal defects/tetralogy of Fallot| 4/20 (20.0)                  | 6/30 (20.0)                     | 10/50 (20.0)   |
| Ventricular septal defect              | 9/20 (45.0)                  | 7/30 (23.3)                     | 16/50 (32.0)   |
| Atrial septal defect                   | 5/20 (25.0)                  | 4/30 (13.3)                     | 9/50 (18.0)    |
| Other defects                          | 10/20 (50.0)                 | 22/30 (73.3)                    | 32/50 (64.0)   |
| Palatal abnormalities                  | 28/29 (96.6)                 | 67/70 (95.7)                    | 95/99 (96.0)   |
| Cleft lip                              | 0/28 (0)                     | 1/67 (1.5)                      | 1/95 (1.1)     |
| Cleft palate                           | 7/28 (25.0)                  | 24/67 (35.8)                    | 31/95 (32.6)   |
| Cleft lip and palate                   | 1/28 (3.6)                   | 12/67 (17.9)                    | 13/95 (13.7)   |
| Submucous cleft                        | 6/28 (21.4)                  | 14/67 (20.9)                    | 20/95 (21.1)   |
| Velopharyngeal insufficiency           | 12/28 (42.9)                 | 22/67 (32.8)                    | 34/95 (35.8)   |
| Cleft uvula                            | 3/28 (10.7)                  | 11/67 (16.4)                    | 14/95 (14.7)   |
| Immunological alterations/ recurrent infections | 17/26 (65.4)                 | 33/55 (60.0)                    | 50/81 (61.7)   |
| Developmental delay                    | 17/31 (54.8)                 | 43/75 (57.3)                    | 60/106 (56.6)  |
| Speech delay                           | 16/31 (51.6)                 | 28/75 (37.3)                    | 44/106 (41.5)  |
| Learning disabilities                  | 23/31 (74.2)                 | 36/75 (48.0)                    | 59/106 (55.7)  |
| Behavioral disturbances                | 9/31 (29.0)                  | 21/75 (28.0)                    | 30/106 (28.3)  |
| Neurologic abnormalities               | 7/22 (31.8)                  | 16/56 (28.6)                    | 23/78 (29.5)   |
| Hearing loss                           | 16/28 (57.1)                 | 20/59 (33.9)                    | 36/87 (41.4)   |
| Sensorineural                          | 13/16 (81.3)                 | 7/20 (35.0)                     | 20/36 (55.6)   |
| Conductive                             | 3/16 (18.8)                  | 10/20 (50.0)                    | 13/36 (36.1)   |
| Ophthalmologic abnormalities           | 8/21 (38.1)                  | 25/59 (42.4)                    | 33/80 (41.3)   |
| Gastroenterological abnormalities      | 10/25 (40.0)                 | 25/57 (37.3)                    | 35/92 (38.0)   |
| Genitourinary abnormalities            | 4/21 (19.0)                  | 11/61 (18.0)                    | 15/82 (18.3)   |
| Skeletal abnormalities                 | 11/30 (36.7)                 | 29/71 (40.8)                    | 40/101 (39.6)  |
| Short stature                          | 8/29 (27.6)                  | 29/73 (39.7)                    | 37/102 (36.3)  |
| Facial dysmorphisms                    | 32/33 (97.0)                 | 66/76 (86.8)                    | 98/109 (89.9)  |
| Long face                              | 22/32 (68.8)                 | 39/66 (59.1)                    | 61/98 (62.2)   |
| Hooded eyelids                         | 13/32 (40.6)                 | 21/66 (31.8)                    | 34/98 (34.7)   |
| Typical nose                           | 21/32 (65.6)                 | 48/66 (72.7)                    | 69/98 (70.4)   |
| Hypertelorism                          | 4/32 (12.5)                  | 18/66 (27.3)                    | 22/98 (22.4)   |

*aFor each listed feature, the denominator represents the number of patients with recorded data.*
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