Molecular Screening for 22Q11.2 Deletion Syndrome in Patients With Congenital Heart Disease

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Abstract Few studies have investigated the prevalence of 22q11.2 deletion syndrome (22q11.2DS) among patients with isolated heart defects or nonconotruncal heart defects. Polymerase chain reaction (PCR) followed by length polymorphism restriction fragment analysis (RFLP) is useful for low-cost molecular diagnosis and screening. This cross-sectional study included 392 patients with congenital heart disease, described clinical features, and performed PCR–RFLP for analysis of polymorphism in three loci with a high heterozygosity rate located in the typically deleted region of 1.5 megabases. Heterozygosity excluded 22q11.2DS. Patients with homozygosity for the three markers underwent multiplex ligation-dependent probe amplification (MLPA) and fluorescence in situ hybridization (FISH) for the final diagnosis, estimating the prevalence of 22q11.2DS. The use of PCR–RFLP excluded 22q11.2DS in 81.6 % (n = 320) of 392 patients. Of the remaining 72 patients, 65 underwent MLPA, showing 22q11.2DS in five cases (prevalence, 1.27 %). Four of these five patients underwent FISH, confirming the MLPA results. All five patients with the deletion had heart diseases commonly found with 22q11.2DS (interrupted aortic arch, persistent truncus arteriosus, tetralogy of Fallot, and ventricular septal defect plus atrial septal defect). Two patients had congenital extracardiac anomaly (one with arched palate and micrognathia and one with hypertelorism). Three patients reported recurrent respiratory infections, and one patient reported hypocalcemia. All were underweight or short in stature for their age. This study contributed to showing the prevalence of 22q11.2DS in patients with any congenital heart disease, with or without other features of the syndrome. Patients with 22q11.2DS may not have all the major features of the syndrome, and those that are found may be due to the heart defect.

Keywords Congenital heart defects · 22q11.2 · 22q11.2 Deletion syndrome · DiGeorge syndrome

Introduction

The 22q11.2 deletion syndrome (22q11.2DS), with an incidence of 1 in every 4,000–5,000 live births [30], is the second leading chromosomal cause of congenital heart disease [16]. Congenital heart diseases, mainly conotruncal, are related to this syndrome in 75 % of cases [29, 37].

The 22q11.2 region contains long stretches of repeated sequences clustered together and known as low-copy repeats (LCRs) sharing more than 95 % of identity. The deletion occurs by nonallelic homologous recombination between these LCRs. Most patients (90 %) had deletions in
the same region of about 3 megabases (Mb) called the DiGeorge syndrome-critical region, which contains about 40 genes [5, 32].

Fluorescence in situ hybridization (FISH), the gold standard test to determine 22q11.2DS, detects more than 95 % of cases but is very expensive, time consuming, and not widely available, precluding its use for widespread screening in settings with limited resources [7, 8]. Recently, studies have shown that polymerase chain reaction (PCR) assays with analysis of restriction fragment length polymorphism (RFLP) based on homozygosity at consecutive markers in the Di-George chromosomal region can be useful in screening for microdeletions with less cost as well as high sensitivity and specificity [8, 11, 14, 26] but that PCR–RFLP should be complemented by FISH [8, 26] or multiplex ligation-dependent probe amplification (MLPA) for the final diagnosis.

The MLPA method is reliable, fast, and economic, with 95–99 % sensitivity and 97–99 % specificity [10, 36]. The advantages of PCR over FISH are agility (because FISH requires cell culture), facility, low cost per sample, small number of deoxyribonucleic acid (DNA) requirements, possibility of using samples with longer storage, and greater sensitivity in screening for 22q11.2 deletion [19].

The prevalence of 22q11.2DS may be greater than known due to the existence of cases with mild or atypical manifestations [24] or nonconotruncal heart defects [38]. Few studies investigating the prevalence of 22q11.2DS have included patients with isolated heart defects who show no other features of the deletion. Early identification of the syndrome allows searching for associated anomalies, provision of genetic counseling, and facilitation of appropriate multidisciplinary management [17].

This study aimed to estimate the prevalence and clinical features of 22q11.2DS in a sample of patients who had congenital heart disease, with or without manifestations of 22q11.2DS, including patients with conotruncal and other congenital heart disease, using PCR–RFLP to exclude the deletion and performing MLPA and FISH in cases for which it was not possible to exclude deletion by PCR–RFLP.

Materials and Methods

This cross-sectional study included 392 patients with congenital heart disease followed at the Pediatric Cardiology Division of Instituto de Cardiologia/Fundação Universitária de Cardiologia (IC–FUC), Porto Alegre, Brazil. These patients were selected by random sampling from January 2007 to May 2008. Patent ductus arteriosus in children younger than 3 months and patent foramen ovale were not considered congenital heart defects. We excluded patients with Down syndrome, Edward’s syndrome, and Patau syndrome, which are strongly associated with congenital heart disease [25]. The study also excluded patients who received blood transfusions in the 4 months before blood collection to avoid contamination of the donor DNA [35].

All the patients or their guardians signed the informed consent before their inclusion in the study. The study protocol was approved by the institutional Ethics Committee and performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

None of the patients who fulfilled the inclusion criteria refused to participate. The patients, their parents, or both were interviewed to obtain information about symptoms, serum calcium, psychomotor development, presence of other congenital anomalies, and family history.

Clinical examination and echocardiography with two-dimensional image and color Doppler were performed. Anthropometric measurements were obtained for verification of percentiles of weight for age and of height for age according to tables from the National Center for Health Statistics/National Center for Chronic Disease Prevention and Health Promotion–2000 [20]. The patients’ charts were reviewed to obtain data about heart disease.

DNA Extraction for Genetic Evaluation

Venipuncture was used to collect 5 ml of blood from each patient. The Lahiri and Nurnberger [21] method was used for DNA extraction. In cases for which new blood collection was necessary, DNA extraction was performed with PureLink Genomic DNA Kits (Invitrogen, São Paulo, Brazil).

PCR–RFLP

To exclude the presence of 22q11.2 microdeletion, PCR–RFLP was performed, with analysis of the polymorphism in rs4819523, rs5748411, and rs4680 loci, which lie in the affected region of typical 1.5 Mb deletion in 22q11.2DS [14]. Reactions were performed in the TC-412 thermal cycler (Techne, Stone, UK) in a final volume of 25 μl using 10 pmol of each primer, 1× PCR buffer, 2 mmol/l of MgCl₂, 0.2 mmol/l of dNTP, and 1 U of Taq DNA polymerase.

All the reagents for PCR were purchased from Invitrogen (São Paulo, Brazil). The quantity of DNA used in each reaction was approximately 200 ng for amplification of loci rs4819523 and rs4680 and approximately 10 ng for amplification of locus rs5748411. The primers used for specific PCR, amplification conditions, and amplicon sizes are shown in Table 1. We designed these primers.

To prove effective amplification of different fragments, PCR products were analyzed by electrophoresis in 1 % agarose gel stained with GelRed dye (Biotium, Hayward, CA, USA). Images were documented in EC3 Imaging Systems (UVP, Upland, CA, USA).
The amplification products were cleaved by the following restriction enzymes according to the manufacturer’s recommendations (Fermentas, Vilnius, Vilnius County, Lithuania): HaeIII (for rs4819523 locus) with cleavage products of 156, 75, 38, and 35 base pairs (bp) in the presence of the cleavage site in the polymorphic locus or 156, 110 and 38 bp in the absence of the cleavage site in the polymorphic locus; BclI (for rs4680 locus) with cleavage products of 102 and 115 bp; and BsrI (for rs5748411 locus) with cleavage products of 64 and 138 bp. The cleavage products were analyzed in 0.75 % agarose with 1.65 % Synergel (BioAmerica, Miami, FL, USA), equivalent to 4 % agarose gel. Samples were stained with GelRed dye (Biotium). The images were documented in EC3 Imaging Systems.

The detection of heterozygosity in the first marker was sufficient to exclude the presence of 22q11.2 microdeletion. In cases of homozygosity, PCR–RFLP was performed for other loci. When homozygosity was detected in the three analyzed loci, the sample was subsequently analyzed by the MLPA method.

MLPA

Samples with homozygosity for the three consecutive PCR–RFLP markers were analyzed by MLPA using SALSA MLPA kit P023-B2 DiGeorge syndrome/VCF (MRC-Holland, Amsterdam, Netherlands) according to the manufacturer’s recommendations. Amplified products were separated by capillary gel electrophoresis in the ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and analyzed using Coffalyser V9.4 Software (MRC-Holland). All analyzes were performed in duplicate.

FISH

Patients with del22q11.2 detected by MLPA together with their parents were subjected to FISH analysis. Metaphase chromosome preparations were obtained from phytohemagglutinin-stimulated lymphocyte cultures according to standard procedures. The FISH procedure was performed on metaphase spreads from peripheral blood lymphocyte cultures. The DiGeorge/VCFS TUPLE1 probe (Cytocell, Cambridge, UK) was used according to the standard manufacturer’s protocol. In each case, 30 metaphase cells and 100 interphase cells were examined. Hybridizations were analyzed with an epifluorescence microscope, and images were captured with a CCD camera. We considered a chromosome region deleted when the FISH signal from the corresponding probe was absent on one of the homolog chromosomes.

Statistical Analysis

The prevalence of 22q11.2DS approximating 1.47 % in patients with congenital heart disease, as observed by Botto et al. [2], was considered in the calculation of the sample size, with a 95 % confidence interval (CI) of 0.3–2.7 %. A total of 392 patients were estimated.

The characteristics of the sample were analyzed by mean or median for quantitative variables and by proportions for qualitative variables. To determine prevalence, 95 % CIs were calculated. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) (SPSS, Chicago, IL, USA).

Results

The age at the time of blood collection for research on 22q11.2DS ranged from 14 days to 66 years (median 10.9 months). The median age at diagnosis of heart disease was 1 month. Of the 392 patients, 15 (3.8 %) had an echocardiographic diagnosis in the prenatal period. Extracardiac congenital anomalies, both isolated or as part of syndromes, were found in 114 patients (29.1 %). The specific congenital heart lesions are described in Table 2.

PCR–RFLP

The sequence of PCR–RFLP tests, their results (homo- or heterozygosity for each locus), and analysis of

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### Table 1 Primers used for specific PCR–RFLP, amplicon sizes, and amplification conditions

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primers</th>
<th>Amplicon size (bp)</th>
<th>Amplification conditions</th>
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<tbody>
<tr>
<td>rs5748411</td>
<td>ATCAGCCACAGAGCAGACC/GCATAGAAAAGCAACTCC</td>
<td>202</td>
<td>50 cycles (95 °C for 40 s; 62 °C for 40 s; 72 °C for 40 s)</td>
</tr>
<tr>
<td>rs4819523</td>
<td>ACAGTGGGGGACATCAAGG/GGAAGCTCATGGGTCTG</td>
<td>304</td>
<td>50 cycles (95 °C for 40 s; 58 °C for 40 s; 72 °C for 40 s)</td>
</tr>
<tr>
<td>rs4680</td>
<td>GTGATTCCAGGGACACCGGC/GCCCTTTTTCGAGTCTGAC</td>
<td>217</td>
<td>50 cycles (95 °C for 40 s; 58 °C for 40 s; 72 °C for 40 s)</td>
</tr>
</tbody>
</table>

*PCR–RFLP* polymerase chain reaction followed by length polymorphism restriction fragment analysis, bp base pairs

*Initial melting step at 95 °C for 5 min and final extension step at 72 °C for 7 min were used in all reactions*
representative PCR–RFLP images are shown in Fig. 1. Analysis of the rs4819523 locus in the 392 patients showed homozygosity for the allele with the cleavage site in 51 patients (40.5 %), homozygosity for the allele without the cleavage site in 21 patients (16.7 %), and heterozygosity in 54 patients (42.9 %), excluding the presence of microdeletion in these 54 patients. Thus, analysis of polymorphism of three loci affected in the 1.5-Mb region with typical deletion (rs4819523, rs4680, and rs5748411) by PCR–RFLP allowed 22q11.2 microdeletion to be ruled out for 320 patients (81.6 %).

MLPA

Of the 72 cases (18.4 %) for which it was not possible to rule out the presence of microdeletion 22q11.2 using PCR–RFLP, 65 were subjected to MLPA, and 22q11.2 microdeletion was found in five patients. The remaining seven patients (who were not subjected to MLPA) were invited to collect blood to improve the DNA sample quality for MLPA, but two of the patients did not accept, and five were not found after an exhaustive search. The prevalence of 22q11.2DS was 1.27 % (5 of 392 patients; 95 % CI, 0.16–2.38 %) (Fig. 1).

FISH

Four of the five patients with 22q11.2 microdeletion detected by MLPA were subjected to FISH. This analysis showed a signal only on the normal homologue chromosome, indicating deletion of band 22q11.2, confirming MLPA results. The fifth patient died of coagulopathy and cardiogenic shock after heart surgery before collection of blood for FISH (Fig. 1).

In one case, FISH was performed for both parents. In two cases, blood samples were available only from the mother. In one case, the parents did not agree to the collection of blood. Metaphase FISH examinations from the parents showed normal results, with no indication of rearrangement involving the chromosome 22q11.2 critical region.

Clinical Features

The clinical features of patients who had confirmed 22q11.2DS are described in Table 3.

Discussion

The current study, conducted in a referral center, evaluated a large number of outpatients with a proportional sample of congenital heart disease using an inexpensive diagnostic approach and found a 22q11.2DS prevalence of 1.27 % (5 of 392 patients; 95 % CI, 0.16–2.38 %). The prevalence would be 1.29 % (5 of 385 patients; 95 % CI, 0.16–2.38 %).
0.16–2.42%) if the seven patients not subjected to MLPA were excluded or 3% (12 of 392 patients; 95% CI, 1.31–4.69%) if all seven of these patients were carriers of the 22q11.2 microdeletion.

This study included patients with all types of congenital heart disease, not only conotruncal disease, with or without extracardiac manifestations of 22q11.2DS. Recent data from the same region of the country using FISH showed a
22q11DS prevalence of 2% in 198 patients with congenital heart disease who were hospitalized in a pediatric intensive care unit (i.e., patients with more severe heart disease than those evaluated in the current study) [28]. A study in India detected SD22q11.2 in 5.7% of 105 patients who had congenital heart disease without extracardiac malformations [12]. Another study, in the United States, detected 22q11.2DS in 1.47% of 2,396 patients with major congenital heart disease [2].

In 2003, Pereira et al. [26] conducted a population-based study in São Paulo, Brazil and concluded that the specificity of the test using three markers was 98.3%. Adding a fourth marker did not achieve a significant increase in specificity. The results showed that PCR–RFLP can be a very good test for exclusion.

We used three DNA markers located in the region of 1.5 Mb, which is deleted in the majority of patients with 22q11.2DS [5, 32]. This approach would not detect only the rare cases of atypical deletions [27]. The PCR method has the disadvantage of failing to detect mosaicism [9], but this is a rare find in 22q11.2DS [18]. It was possible to exclude the presence of 22q11.2 microdeletion by PCR–RFLP in 81.6% of the patients, leaving few patients for the use of MLPA. As a confirmatory test, this approach was fast, accurate, and cost effective, showing concordance with FISH findings in studies investigating 62 patients [36], 30 patients [10], and 51 patients [33] with 22q11.2DS.

Previous studies demonstrated that the spectrum of clinical features in 22q11.2DS is very varied [24] and that individuals with similar molecular defects show clinical variability, which seems unrelated to the size of the deleted region [1]. More than 180 clinical features in 22q11.2DS have been described [29, 37]. Immunodeficiency occurs in 40–93%, hypoparathyroidism in 40–60% [23], and palatal abnormalities in 69% [4] of patients with 22q11.2DS. Neurologic and psychiatric disorders also may occur [6, 13].

Congenital heart disease may be nonconotruncal [29, 38]. According to reports, hypoparathyroidism may manifest only after cardiac surgery [31]. In our study, patients in the 22q11.2DS group showed varied features of the syndrome. Only one of the five patients had previous suspicion of the deletion. This patient had interrupted aortic arch, a heart defect in which 50% of the patients have the deletion [2]. All five patients had heart diseases commonly found with this syndrome [29], but they did not have all the major features of the 22q11.2DS. At physical examination, only two had congenital extracardiac anomalies, and these abnormalities were not pathognomonic features of the 22q11.2DS. No cases of hypoparathyroidism were found, but asymptomatic patients may not have been identified because parathyroid hormone was not systematically evaluated.

The reported respiratory infections could have been due to heart disease or primary or secondary immunodeficiency due to another cause. Poor weight or height gain could have been due to heart disease and its therapeutic interventions, not necessarily 22q11.2 microdeletion. However, according to Brauner et al. [3], short stature may be due to growth hormone deficiency in patients with 22q11.2DS. Developmental delay also could be due to heart disease or its interventions, as suggested by other authors [22]. However, Van Aken et al. [34] showed motor deficits and a negative effect on the intelligence quotient without the effect of congenital heart disease in the group of children with 22q11.2DS compared with the control group. Nonetheless, in our study, seizures were reported by 11.5% of the patients, which is much higher than the incidence in the general population (1–3%) [15].

In conclusion, PCR–RFLP for screening of 22q11.2DS in patients who have any congenital heart disease, with or without other features of the syndrome, allows the presence of deletion in most patients to be excluded at low cost, and the final diagnosis can be established by a complementary method such as FISH or MLPA. Patients with 22q11.2DS may not have all the major features of the syndrome, and those that are found may be due to the heart defect or other causes, delaying the early diagnosis of other abnormalities and genetic counseling. This study contributed to showing a more real prevalence of 22q11.2DS in patients with congenital heart disease because few studies have investigated the prevalence of 22q11.2DS in patients who have any heart defects, not only conotruncal defects, with or without other features of the syndrome.

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References

