Experimental and Molecular Pathology 101 (2016) 116–123

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Post-mortem cytogenomic investigations in patients with congenital malformations

Alexandre Torchio Dias [a,*], Évelin Aline Zanardo [a], Roberta Lelis Dutra [a], Flavia Balbo Piazzon [a], Gil Monteiro Novo-Filho [a], Marilia Moreira Montenegro [a], Amom Mendes Nascimento [a], Mariana Rocha [a,b], Fabricia Andreia Rosa Madia [a], Thais Virgínia Moura Machado Costa [a], Cintia Milani [a], Regina Schultz [c], Fernanda Toledo Gonçalves [d], Cintia Fridman [d], Guilherme Lopes Yamamoto [e], Débora Romeo Bertola [e], Chong Ae Kim [e], Leslie Domenici Kulikowski [a,b]

[a] Department of Pathology, Cytogenomic Laboratory – LIM 03 – HC-FMUSP, Universidade de São Paulo, SP, Brazil
[b] Human Reproduction and Genetic Center, Department of Coletive Health - Faculdade de Medicina do ABC, Santo André, SP, Brazil
[c] Division of Pathology - Clinical Hospital – HC -FMUSP, Universidade de São Paulo, SP, Brazil
[d] Department of Legal Medicine – HC-FMUSP, Universidade de São Paulo, SP, Brazil
[e] Genetics Unit, Instituto da Criança HC-FMUSP - Universidade de São Paulo, SP, Brazil

ARTICLE INFO

Article history:
Received 28 September 2015
and in revised form 16 June 2016
Accepted 19 July 2016
Available online 20 July 2016

Keywords:
Congenital malformations
Post-mortem cytogenomic investigation
DNA copy number variations

ABSTRACT

Congenital anomalies are the second highest cause of infant deaths, and, in most cases, diagnosis is a challenge. In this study, we characterize patterns of DNA copy number aberrations in different samples of post-mortem tissues from patients with congenital malformations. Twenty-eight patients undergoing autopsy were cytogenomically evaluated using several methods, specifically, Multiplex Ligation-dependent Probe Amplification (MLPA), microsatellite marker analysis with a MiniFiler kit, FISH, a cytogenomic array technique and bidirectional Sanger sequencing, which were performed on samples of different tissues (brain, heart, liver, skin and diaphragm) preserved in RNAlater, in formaldehyde or by paraffin-embedding. The results identified 13 patients with pathogenic copy number variations (CNVs). Of these, eight presented aneuploidies involving chromosomes 13, 18, 21, X and Y (two presented inter- and intra-tissue mosaicism). In addition, other abnormalities were found, including duplication of the *TYMS* gene (18p11.32); deletion of the *CHL1* gene (3p26.3); deletion of the *HIC1* gene (17p13.3); and deletion of the *TOM1L2* gene (17p11.2). One patient had a pathogenic missense mutation of g.8535C > G (c.746C > G) in exon 7 of the *FGFR3* gene consistent with Thanatophoric Dysplasia type I. Cytogenomic techniques were reliable for the analysis of autopsy material and allowed the identification of inter- and intra-tissue mosaicism and a better understanding of the pathogenesis of congenital malformations.

1. Introduction

Molecular karyotyping is an important approach for diagnosing malformed newborns and can be performed on material from blood, amniocentesis, skin fibroblasts, chorionic villi, cord blood or a buccal smear. However, genomic copy number investigation on autopsy material, due to technical limitations, is a challenge (Costa et al., 2015).

The use of combined cytogenomic methods could be an alternative to access the molecular karyotype from autopsy tissue and to improve knowledge of the etiology of underdiagnosed congenital malformations. Moreover, postmortem study allows access to different tissues and can enable a more accurate molecular characterization of tissue mosaicism (Gniady et al., 2010; Wapner, 2010).

MLPA is an alternative technique for detecting chromosomal aneuploidies in the genome, assessing copy number differences in approximately 40 genomic regions in a single experiment. MLPA also identifies pathogenic copy number variations (CNVs) based on a diagnostic hypothesis (Christofolini et al., 2010; Dutra et al., 2012). Chromosomal array analysis is an additional method of measuring gains and losses of DNA throughout the human genome and is a first tier test in the genetic evaluation of infants and children with unexplained congenital anomalies (Biesecker and Spinner, 2013).

The Microsatellite Marker Method (MM) is a widely used method for investigating genomic changes, for paternity testing and for forensic analysis and is an alternative approach when the DNA is of poor quality or at low concentrations. MM builds on the analysis of small DNA sequence repeats (STR - Short Tandem Repeats) and enables the

* Corresponding author at: Cytogenomics Laboratory, Department of Pathology, Universidade de São Paulo, Av. Dr. Enéas de Carvalho Aguiar, 155, 2° andar, bloco 12, sala 7, São Paulo, SP, Brazil.
E-mail addresses: alexandre-dias@usp.br, dias.torchio@gmail.com (A.T. Dias).

http://dx.doi.org/10.1016/j.yexmp.2016.07.003
0014-4800/

identification of subjects, the assessment of the origin and number of alleles between different tissues and the study of twin zygosity (mono- or dizygotic) (Hughes-Stamm et al., 2011).

Here, we report the investigation of copy number aberrations using cytogenomic techniques in postmortem tissues from patients with congenital malformations.

2. Materials and methods

2.1. Patients

A total of 28 patients with congenital malformations from non-consanguineous and healthy families were evaluated in the present study. Five different types of tissues, preserved in RNAlater, in formaldehyde or paraffin-embedded, were selected for DNA extraction and molecular investigation. The tissues selected were the brain, heart, liver, skin and diaphragm.

2.2. DNA extraction from tissues

DNA was isolated from tissues using the DNeasy Blood & Tissue Kit® (Qiagen, Valencia, CA) according to the manufacturer's protocol. The integrity of the DNA was evaluated by spectrophotometry (NanoView® - GE Healthcare) and by electrophoresis on 2% agarose gels.

2.3. Multiplex ligation-dependent probe amplification (MLPA)

The MLPA assay was performed with the P095 kit (Aneuploidy probe mix), which encompasses 36 targets on 5 chromosomes (21, 18, 13, X and Y), the P064 kit (Mental Retardation-1 probe mix) and the P070 kit (Human Telomere-5 probe mix); all kits were from MRC Holland®. The steps of DNA denaturation, hybridization of MLPA probes, ligation and PCR reactions were performed according to the manufacturer's instructions with a minor modification– using a smaller amount of genomic DNA. The separation of amplification products by electrophoresis was performed using an ABI 3500 Genetic Analyzer (Applied Biosystems®), and the data were analyzed using GeneMarker® software, version 1.6 (www.softgenetics.com - Softgenetics, LLC, State College, PA, USA). The peak area of each fragment was compared to that of a control sample, and the results were considered abnormal when the relative peak height ratio was below 0.70 (deletion) or above 1.30 (duplication). The details of the regions detected by each kit can be found at www.mlpa.com.

2.4. Microsatellite marker method

DNA samples were amplified in a thermocycler block 9700 with silver using a Minifiler™ Kit (Applied Biosystems), according to the manufacturer's instructions. Amplified products were separated using an ABI 3130 sequencer (AppliedBiosystems), and analysis and DNA profiling were performed using GeneMapper ID™ version 3.2 software (Applied Biosystems), according to protocols provided with the panels and by Applied Biosystems. Minifiler is composed of 9 Short Tandem Repeats (STRs) (D13S317, D7S820, Amelogenin, D2S1338, D21S11, D16S539, D18S51, CSF1PO, and FGA), which include analyzable regions of the chromosomes (that is, compatible with life) related to the major aneuploidies Trisomy 21, Trisomy 18, Trisomy 13 and Monosomy X.

2.5. Fluorescent in situ hybridization

FISH analysis was performed using the following commercial probes (according to clinical evaluation and molecular results): LPS 008 EWSR1/ERG *Translocation*, *Dual Fusion* Probe® and LPE 0XYc - *Dual Labelled Fusion* Probe® (Cytocell, UK) on 4 μm histological sections of tissue, according to Pinkel et al. (1986) with minor modifications.

2.6. Array

The bead-array was performed using the Illumina BeadChip 850 K® array. This product contains approximately 850,000 empirically selected single nucleotide polymorphisms (SNPs) spanning the entire genome but with enriched coverage for 3262 genes of known relevance in CNV aberrations (http://www.illumina.com/products/infinium-cytosnp-850k). The data were analyzed using BlueFuse™ Multi Analysis software. The genomic positions were mapped to the GRCh37/hg19 genome build.

2.7. Sanger sequencing

Sanger sequencing was used to study tissues from patient 28, who was suspected to have Thanatophoric Dysplasia type I (TDI). The most common TDI missense mutations are located in exon 7 of the *FGFR3* gene (p.R248C and p.S249C). The molecular investigation was undertaken using Sanger bidirectional sequencing. DNA was amplified according to a standard laboratory protocol (annealing temperature of 62 °C for 35 cycles, 5% DMSO), including flanking exonic regions. The PCR product was assessed on a 2% agarose gel and purified with ExoSap enzyme (Affymetrix). The product of the reaction was sequenced using an automated DNA sequencer, the ABI Prism Genetic Analyzer ABI3500 (Applied Biosystems). The results were analyzed using Sequencher 5.3 software and the Mutation Taster database.

3. Results

The clinical manifestations in patients with genomic abnormalities and normal results are shown in Table 1.

Genomic abnormalities were found in 13 of the cases, whereas aneuploidies of chromosomes 13, 18, 21, X and Y were present in 8 patients. Of these, two patients showed inter- and intra-tissue mosaicism (Figs. 1 and 2). We found different pathogenic CNVs, including duplication of the *TYMS* gene (18p11.32), deletion of the *CHL1* gene (3p26.3), deletion of the *HIC1* gene (17p13.3) and deletion of the *TOM1L2* gene (17p11.2). One of the cases presented a pathogenic missense mutation of g.8535C > G (c.746C > G) in exon 7 of the *FGFR3* gene, compatible with Thanatophoric Dysplasia type I. The results are shown in Table 2.

4. Discussion

The cytogenomic abnormalities identified by different methodologies were associated with the clinical phenotypes, based on information from a clinically relevant genomic database. Genomic variation and somatic mosaicism involving trisomies of chromosome 21, 13, or 18 or sex chromosome aneuploidy are well-established causes of multiple congenital anomalies that could lead to neonatal death. Nevertheless, the efficient detection of these genomic alterations may be challenging in cases of low-level mosaicism or intercellular variations.

Post-mortem cytogenomic tissue analysis allowed the identification of mosaic, intra- and inter-tissue aneuploidies. The combined analysis using different molecular methodologies certified the accurate identification of different genomic constitutions and cell line distribution. MLPA and MM are first tier methods that can reveal tissue mosaicism, whereas FISH and array approaches can confirm the results. Differences between array and FISH results found in patient 2 can be explained in part by the concomitant presence of abnormal and normal cells.

The search for genomic regions of the Y chromosome, particularly the SRY gene, in patients with monosomy X, is a routine procedure in vivo. It is important for the purposes of clarifying the diagnosis and for prophylactic clinical management, because when the presence of Y chromosomal material is confirmed in cell lines, gonadectomy may be recommended due to the risk of gonadal malignancy (Bianco et al., 2006). This finding in post-mortem samples, such as in patient 16, is rare in the literature. Until now, there has not been enough scientific