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Chromosomal Structural Rearrangements: Characterizing Interstitial Deletions and Duplications in The Clinical Practice

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Abstract

Structural chromosomal rearrangements are traditionally diagnosed based upon clinical manifestations and conventional chromosome analyses. Cytogenetic analysis through GTG-banding in combination with a clinical evaluation was sufficient to detect the genomic imbalances presented here. However, several studies have demonstrated the importance of break-points delimitation and the extent of the deleted and/or duplicated region for the better definition of clinical implications, prognosis and genotype- phenotype correlation. Refined molecular techniques, such as Fluorescence *In Situ* Hybridization (FISH) and array based Comparative Genomic Hybridization (array-CGH), are required for the characterization of chromosomal structural rearrangements, for more precise analysis of break points, for confirmation of critical regions and for the evaluation of new alterations not previously detected. We report here the detection of 4 interstitial deletions and duplications associated with congenital malformations and/or neurological abnormalities, highlighting how cytogenetic technologies allow the expansion of chromosome rearrangements characterization in the clinical practice.

Keywords: Array-CGH; Deletions; Duplications; FISH; Multiple Congenital Abnormalities; Structural Rearrangements

Introduction

Chromosomal structural rearrangements can result from different mutational mechanisms, including DNA recombination-, replication- and repair-associated processes. Thus, Structural Variants (SVs) arise from improperly repaired DNA Double- Strand Breaks (DSB). DSBs are a frequent occurrence in all classes and two major pathways are involved in their repair: homologous recombination and non-homologous end joining. However, homologous recombination appears to be the predominant pathway underlying recurrent rearrangements of our genome [1]. Errors on during these repair mechanisms can result in SVs that involve losses, gains and rearrangements ranging from a few nucleotides to entire chromosomal arms. Factors such as rearrangements, hotspots and induced DSBs (Double-Strand Breaks) are implicated in the formations of SVs [2,3]. Current approaches to understand the formation of chromosomal structural rearrangements in the human genome include the direct observation of human genomic alterations, or rearranged end products, that convey a disease trait. The study of disease-causing structural rearrangements, as chromosomal deletions and duplications, provides an opportunity to explore the genomic mechanisms leading to such events. In addition to identify genomic structural rearrangements, several studies have revealed complex exonic, genic and chromosomal rearrangements that can be generated in a single mutagenic event [4-6]. Other studies have shown that the formation of structural rearrangements can be accompanied by additional genome modification that may result in a disease trait [7-10]. The prediction of phenotypic consequences

of some types of chromosomal rearrangements remains difficult, partially owing to our incomplete knowledge of genes that are haploinsufficient (in which half a dose is detrimental) or dosage sensitive (in which both increased and decreased gene dose is detrimental) [11]. Therefore, the use of combined molecular analytic tools is necessary to delineate the entire range of variation that is associated with a chromosomal structural rearrangement in an individual personal genome. New mechanistic discoveries in humans are elucidating how the formation of chromosomal structural rearrangements can re-structure a specific region of the genome to change gene expression either locally or genome-wide [12,13] with pathological consequences for carriers [14].

Structural features of particular genomic regions can trigger the formation of recurrent and non-recurrent chromosome rearrangements, including chromosomal deletions and duplications. Those can be either interstitial or terminal. An interstitial chromosome deletion or duplication refers to the lost or gain of a segment of DNA in the middle of a chromosome. A terminal deletion or duplication refers to a region that is from the telomere of either short arm or the long arm of a chromosome to a lost/gain region within the chromosome. Recurrent rearrangements share a common genomic size with clustered breakpoints and non-recurrent rearrangements visible by light microscopy are distributed throughout the genome. They have different sizes and distinct breakpoint combinations. Although there is some overlap, the two groups of structural abnormalities display clear differences in how and when they arise [15].

For recurrent rearrangements, the predominant mechanism is Nonallelic Homologous Recombination (NAHR) mediated by Low Copy Repeats (LCRs, also called segmental duplications or SDs) [16]. NAHR is a reciprocal process resulting in the gain or loss of the genomic region flanked by the LCRs and is responsible for a large number of genomic disorders including e.g, Digeorge Syndrome (DGS) and Williams-Beuren syndrome (WBS). NAHR may involve both chromosome homologues (interchromosomal) or separate chromatids of only a single chromosome (intrachromosomal). An interchromosomal origin is likely to indicate a meiotic event while an intrachromosomal origin may be either meiotic or mitotic [16-18]. Recurrent microdeletions and microduplications are mainly interchromosomal and are assumed to arise at meiosis [17,18]. Although more frequent, the formation of non-recurrent rearrangements is less well understood. Among cytogenetically visible deletions and duplications, there appear to be approximately equal numbers of interchromosomal and intrachromosomal abnormalities although only relatively small numbers have been investigated [19]. Until recently, it was assumed that most non-recurrent imbalances arise through Non-Homologous End Joining (NEHJ), a process that joins double-stranded breaks in the absence of extensive homologous sequence [16]. However, alternative mechanisms have been proposed, such as Fork Stalling and Template switching (FoSTes) [8] and Microhomology-Mediated Breakpoint-Induced Replication (MMBIR) [20]. These are mitotic mechanisms based on stalling of the replication fork during DNA replication.

Improvements in classical and molecular cytogenetic techniques over the past 40 years have allowed an increasingly sensitive detection of chromosomal rearrangements, including deletions and duplications related to genomic disorders [21]. Cytogenetic and cytogenomic methods that can be combined in the routine analysis, include karyotyping, Fluorescence *In Situ* Hybridization (FISH), array Comparative Genomic Hybridization (aCGH) and SNP arrays [22]. Therefore, the aim of this study was to characterize interstitial chromosomal segmental deletions or duplications. In addition, this study explores the types of genomic rearrangements leading to such imbalances and the contribution of cytogenomic methodologies to further characterize chromosomal structural rearrangements in the clinical practice.

Material and Methods

Sample Selection

This retrospective study was performed using DNA extracted from blood samples of 4 subjects with Multiple Congenital Abnormalities (MCAs) and interstitial structural imbalances investigated at the Clinical Genetics section, Hospital Materno Infantil Presidente Vargas (HMIPV), Brazil is a regional pediatric referral center of the state of Rio Grande do Sul, in South Brazil. Venipuncture was used to collect 5 ml of blood from each patient. The Lahiri and Nurnberg method was used for DNA extraction. In cases for which the collection of a new blood sample was necessary, DNA extraction was performed with a Pure Link Genomic DNA kit (Invitrogen, São Paulo, Brazil). The subjects presented ages between 8 months and 11 years at the time of blood collection. Their clinical and laboratory data from the first referrals were collected from the hospital's records. The data did not include follow-up investigations or disease outcomes. The study was carried out with 4 samples with interstitial structural rearrangements. From the 4 samples, 3 presented deletions and 1 a duplication. All patients' guardians provided informed consent before their inclusion in the study. Parental studies were performed, to determine whether the findings represent *de novo* or familiar events. The study protocol was approved by the Institutional Ethics Committee and was conducted in accordance with current institutional ethics rules regarding the use of biological materials from biorepositories [23].

Molecular and Classical Cytogenetic Studies

Peripheral blood lymphocytes from each patient and parents were cultured for 72 hours according to standard procedures. Cytogenetic analysis was performed on GTG- banded chromosomes, and 20 cells from the patients and 20 cells from each parent were fully investigated. that were found to be rearranged by karyotype, centromere or locus-specific probes were hybridized to methaphases and interphases of patients and parents. FISH ex-

periments were carried out in all cases through standard techniques using commercially available locus-specific probes and centromeric-probes (Abbott Laboratories, Des Planes, Illinois, EUA and Cytocell, Cambridge) for the following regions: D18Z/18p11.1-q11.1, BCL2/18q21.33, subtelomere 18p/18p11.32, subtelomere 18q/18q23, MYC/8q24.21 and GSDMC/8q24.21.

Hybridizations were analyzed with an epifluorescence microscope, and images were captured with a charged-couple device camera. At least 30 cells were analyzed per hybridization. We considered a chromosome region deleted when the FISH signal from the corresponding probe was absent from one of the homologous chromosomes. A chromosome duplication region was considered when at least three signals from the corresponding probe were observed.

Array Comparative Genomic Hybridization (array-CGH)

We performed comparative genomic analysis with oligonucleotide array-based CGH using an 8x60K whole-genome platform (design 021924, Agilent Technologies, Santa Clara, California, United States of America), with an average distance of 40 kb between probes. Genomic DNA was isolated from the peripheral blood of 4 subjects (available at the biorepository) and subsequently analyzed. For each experiment, a gender-mismatched normal reference (Promega Corp., Madison, WI, USA) was used. The experiments were performed according to the manufacturer's protocol. Images of arrays were taken using a microarray scanner (G2600D) and processed using the Feature Extraction software (v 9.5.1), both from Agilent. For the measurement of data quality, various quality control (QC) parameters have been employed, and included in software packages both from commercial sources and from public domain. These QC parameters calculate microarray data noise, an appreciation which is critical to some assessments, like false discovery rate. For those CGH arrays manufactured by Agilent Technologies, as used in our study, the major QC parameter is known as the derivative log ration, or DLR. In our study, DRL values of <0.15 were indicative of acceptable data. The raw data were analyzed using the Agilent Cytogenomics v2.7.8.0 software and the ADM-2 statistical algorithm (second generation algorithm that can assist in controlling noise source), with a threshold of 6.0 and a 4-probe minimum aberration call. Subsequent normalization of the data was performed using the software to verify changes in DNA copy number. The P-values for each probe were calculated, providing additional objective statistical criteria to determine if each probe's deviation from zero was statistically significant. All experiments included two array hybridizations per sample, and results were recorded and compared. To exclude false positive results, we confirmed the detected deletions and duplications using dye-swap experiments. Laboratory personnel were blinded until test results became available. Array-CGH detected all known regions of genomic imbalance in the 4 validation samples with a concordance of 100% and an excellent signal-to-noise ratio (<0.1). Only genomic imbalances that were detected in both dye-swap experiments were reported.

Array-CGH and genomic data analysis

Whole-genome array-CGH data analysis was performed in a blinded fashion. Specifically, the samples were obtained, the identifying information was removed, and investigators who performed the array-CGH analyses were not aware of the prior clinical and/or laboratory information related to each sample. The deletions and duplications segments that were detected were compared with losses and gains that had been reported in at least 3 publicly available online resources and in databases of chromosomal abnormalities and variants. Our own in-house database was also consulted. As a reference, we used public data from compiled, collaborative databases, including the Clinical Genomic Resource (ClinGen) (http://clinicalgenome.org/); the Database of Chromosomal Imbalance and Phenotype in Humans Using Ensembl Resources (Decipher) (http://decipher.sanger.ac.uk/); the European Cytogeneticists Association's Register of Unbalanced Chromosome Aberrations (ECARUCA) (http://umcecaruca01.extern.umcn. nl:8080/ecaruca/ecaruca.jsp); the Ensembl Genome Browser (http://www.ensembl.org/index.html); the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/); and the University California Santa Cruz (UCSC) Genome Browser (http://genome.ucsc.edu/). A review of genes and other loci mapped to the deleted and duplicated region was performed, using human genome databases, such as the Online Mendelian Inheritance in Man (OMIM®).

For every imbalance, the UCSC database was also used to look for the presence of LCRs in the breakpoint intervals mapped by array-CGH. The breakpoint interval was defined by the maximum and minimum size of the imbalance, and ranged in size from 5 to 250 kb according to the array platform used and the density of probes in that genomic region. The origin of an imbalance was assumed to have been mediated by NAHR if paralogous LCRs spanned all or a large proportion of both breakpoint intervals, while an imbalance was assumed to have arisen by a mechanism other than the NAHR if LCRs were absent from the breakpoint intervals. The orientation of the LCR was not taken into consideration.

Results and Discussion

The aim of this study was to retrospectively characterize interstitial chromosomal segmental deletions or duplications using cytogenomic methods in samples available from subjects with multiple congenital abnormalities.

The data of the chromosomal structural rearrangements associated with congenital anomalies, whose cells in suspension and DNA samples were analyzed, are presented in Table 1. This study characterized 3 cases with interstitial deletions (del3q11.2-q13.31;

del6p24.3-p22.3; del18q21.2-q23) and 1 case with an interstitial duplication (dup8q24.13-q24.3). From those cases, 3 were female and 1 was male. The details of the array-CGH results from the genomic imbalances identified are summarized in Table 1. The extension of deletions and duplications analyzed ranged between 9.62 Mb (del6p24.3-p22.3), 16.44 Mb (del3q11.2-q13.31), 17.18 Mb (dup8q24.13-q24.3) and 26.61 Mb (del18q21.2-q23). Overall, 2 *de novo* deletions were verified (del6p24.3-p22.3; del18q21.2-q23) and further inherited deletion and inherited duplication where verified in 2 samples (del3q11.2-q13.31 and dup8q24.13-q24.3). Additionally, a FISH test confirmed the array-CGH results in 2 samples from which stored cells were available (Figures 3D, E and Figure 4C).

Case	Gender/ age*	Del/ Du p	Chromo-some Region	Orienta-tion	Genomic coordinates (hg 19)	Size (Mb)	Origin	Associated Clinical features*
1	F/6 y	del	3q11.2-q13.31	dir	97,791,743-114,234,782	16.44	mat	Microcephaly, Up-Slanting And Oblique Palpebral Fissures, Broad Nasal Root, Bulbous Nasal Tip, High Palate, Astigmatism, Patent Ductus Arteriosus, Cholestasis, Speech Delay.
2	F/8m	del	6p24.3-p22.3	dir	9,734,048-19,355,673	9.62	dn	Growth Retardation, Cerebral Hypoplasia, Microphthalmia And Nystagmus (Left Eye), Strabismus, Submucosal Cleft Palate with Unilateral Cleft Lip (Right), Severe Micrognathia, Atrial and Ventricular Septal Defect, Clubfoot (Left).
3	F/3y	del	18q21.2-q23	inv	52,475,229-78,858,446	26.61	dn	Growth Retardation, Microcephaly, Cerebral Hypoplasia, Bitemporal Narrowing, Epicanthus, Long Thumbs, Sacral Dimple, Hypotonia, Psychomotor And Speech Delay, Stereotyped Movements.
4	M/8 y	dup	8q24.13-q24.3	inv	125,385,074 -142,496,610	17.18	mat	Microcephaly, Low-Set Hair On The Forehead, Long Eyelashes, Micrognathia, Hemangioma In The The Lower Lip, Seizures, Speech Delay.
*age at the time of clinical evaluation; del: deletion; dir: direct; dn: *de novo*; dup: duplication; inv: inverted; F: female; M: male; m:months; y:years; mat: maternal								

Table 1: Details of the genomic imbalances analyzed in patients with interstitial chromosome rearrangements.

Among 3 samples with deletions, one case exhibited a visible cytogenetically interstitial deletion of chromosome 3 identified through G-banding, involving the proximal segment of chromosome 3 at band q11.2 (Figure 1A). The paternal karyotype was normal. The deletion was identified as inherited from the mother, who had the same deletion detected through karyotypical analysis (data not shown). The precise characterization of the deletion using oligonucleotide aCGH in both, child and mother, showed a loss of genomic material corresponding to an interstitial deletion in the long arm of chromosome 3, segment 3q11.2-q13.31, of approximately 16.44 Mb (UCSC Genome Browser on Human Dec. 2013 (GRCh38/hg38). Arr 3q11.2-q13.31 (97,791,743- 114,234,782) ×1 (Figure 1B). It is evident already from the karyotype analysis that a large chromosome deletion would involve many genes and be causally related to the congenital anomalies observed in both proband and mother.

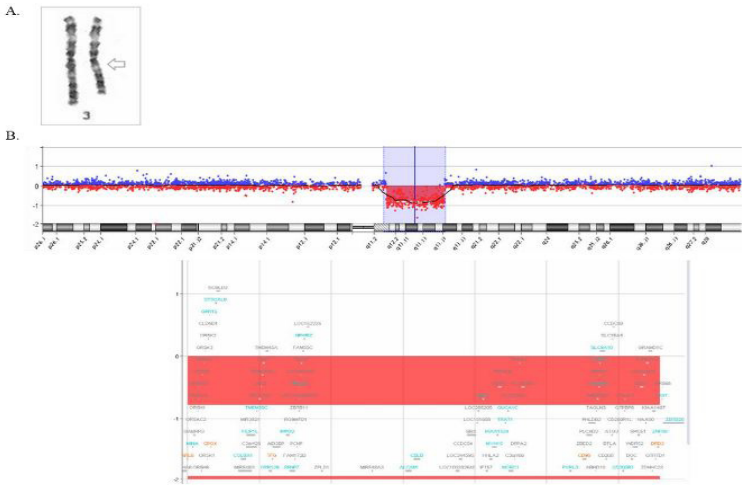

Figure 1(A-B): (A): Chromosome banding shows an interstitial deletion on chromosome 3q11.2. (B): Array-CGH ratio profiles of chromosome 3q11.2-q13.31 using genomic DNA from the patient as a test (Red) and DNA from normal subjects as a reference (Blue). The test/reference ratio data for the chromosome 3 is shown. Each dot represents a single probe (Oligo) spotted on the array. The log ratio of the chromosome probes is plotted as a function of chromosomal position. A copy number loss shifts the ratio downward (approximately -1x). The ideogram of chromosome 3 profile shows the location of each probe. The probe log2 ratios were plotted according to genomic coordinates (based on the UCSC Genome Browser, December 2013, NCBI Build 38 reference sequence). Top, chromosome 3 array-CGH profile indicating the interstitial deletion on the long arm (Blue Box); bottom, detail of the 3q11.2-q13.31 gene region showing the deletion of approximately 16.44-Mb from sample 1 (Red Line).

The proband at the age of 6 years presented at the clinical evaluation, microcephaly, oblique palpebral fissures upward, broad nasal root, bulbous nasal tip, high palate, cholestasis, patent ductus arteriosus and speech delay (Table 1). Her mother at the age of 28 years has ocular hypertelorism, oblique palpebral fissures upward, midfacial hypoplasia, hypoplasia of nose wings, missing teeth (she said teeth were changed and made use of denture since 15 years old), triangular neck, narrow thorax, lumbar concavity and fingernails with subungual detachment and learning difficulties.

Although the phenotypic features related to sample 1 is within the spectrum of the clinical features associated with partial or entire deletions of the 3q11.2-q13.31 region, most of the cases present intellectual disability, speech delay and epicanthus with no case of cholestasis associated reported. The cholestasis finding associated with the sample 1 was firstly suggestive of the diagnosis of α-1-Antitrypsin Deficiency (AATD) [24], a condition with an autosomal codominant pattern that may cause lung disease and liver disease affecting about 1 in 1,500 to 3,500 individuals with European ancestry. Thus, in this situation, other genetic variant along with this deletion is probable contributing to variable expressivity and penetrance observed in this family. Unfortunately, maternal DNA was not available to assess further characterization of the deletion segregating in this family. Bridging the knowledge between the inherited deletion identified here and phenotype requires more precise structural variant information and the identification of the elements that are responsible for the observed trait.

The contiguous deletion on chromosome 6p24.3 investigated in sample 2 was associated with dysmorphic features (Table 1) noted in a patient referred to the clinical genetics section at age of 8 months, including growth retardation, cerebral hypoplasia, microphthalmia and nystagmus (Left Eye), strabismus, submucosal cleft palate with unilateral cleft lip (Right), severe micrognathia, atrial and ventricular septal defect and unilateral clubfoot (Left). The interstitial deletion spans 9.62 Mb from 6p24.3 to p22.3 (Figure 2B). Array 6p24.3-p22.3 (9,734,048-19,355,673) ×1. This region contain more than 29 genes, including TFAP2A (implicated in Branchio-Oculo-Facial Syndrome [25-27] and partially OFC1 (Orofacial Cleft 1) genetic locus. Branchio-oculo-facial syndrome (BOFS, OMIM# 113620) is a rare autosomal dominant disorder characterized by branchial cleft sinus defects, ocular anomalies and facial dysmorphisms, including lip or palate cleft or pseudocleft, and is associated with mutations in the TFAP2A gene.

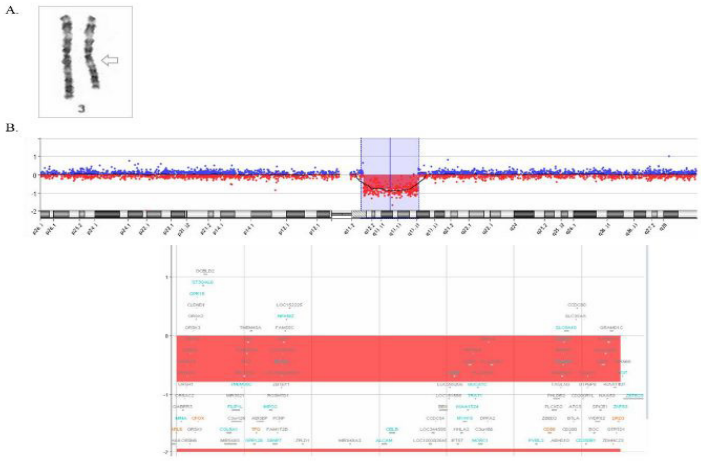

Figure 2(A-B): (A): Chromosome banding shows an interstitial deletion on chromosome 6p24. (B): Array-CGH ratio profiles of chromosome 6p24.3-p22.3 using genomic DNA from the patient as a test (Red) and DNA from normal subjects as a reference (Blue). The test/reference ratio data for the chromosome 6 is shown. Each dot represents a single probe (Oligo) spotted on the array. The log ratio of the chromosome probes is plotted as a function of chromosomal position. A copy number loss shifts the ratio downward (approximately -1x). The ideogram of chromosome 6 profile shows the location of each probe. The probe log2 ratios were plotted according to genomic coordinates (based on the UCSC Genome Browser, December 2013, NCBI Build 38 reference sequence). Top, chromosome 6 array-CGH profile indicating the interstitial deletion (blue box); Bottom, detail of the 6p24.3-p22.3 gene region showing the segment deletion of approximately 9.62-Mb from sample 2 (Red Line).

In most cases reported, the mutations involving TFPA2A result in BOFs and only sporadic cases show cryptic chromosome abnormalities. Dumitrescu [28], detected a 3.2 Mb cryptic chromosomal deletion in an affected mother and her son with BOFS at chromosome 6p24.3. These genomic alterations, even when found in a small percentage of cases, can significantly narrow the candidate region and allow successful discovery of the candidate genes. Although we report a larger deletion of 9.62 Mb involving TFPA2A and other contiguous genes as well. Probable TFPA2A gene is responsible for many aspects of the phenotype associated with the del6p24.3-p22.3 evaluated in this work.

A previous karyotype analysis of sample 3 revealed the presence of a suggestive terminal deletion on the long arm of chromosome 18 (Figure 3A). The parents of the index case had a normal karyotype, FISH and array-CGH analyses (Data not Shown). Array- CGH analysis from the proband allowed for additional genomic information regarding the previously identified deletion and the characterization of an interstitial deletion spanning 26.61 Mb in the long arm of chromosome 18, segment q21.2-q23 (Figure 3B). Array 18q21.2-q23 (52,475,229-78,858,446) ×1. FISH analysis with the BCL2/18q21.33 region probe confirmed the deletion of the critical region identified by array-CGH (Figure 3D). Furthermore, FISH with the 18 centromere/DXZ1 probe, subtelomere/18p11.32 probe and subtelomere 18q/18q23 probe revealed the occurrence of an inversion of the long arm of chromosome 18 (Figure 3E). FISH analyses of the parental chromosomes revealed normal result with the same FISH probes used, thus excluding a familial deletion and/or inversion of the corresponding region (Data Not Shown).

Figure 3(A-E): (A): Chromosome banding shows a deletion on chromosome 18q21. (B): Chromosome 18 array-CGH profile indicating an interstitial deletion (Blue Box). The ideogram of chromosome 18 profile shows the location of each probe. The probe log2 ratios were plotted according to genomic coordinates (based on the UCSC Genome Browser, December 2013, NCBI Build 38 reference sequence). (C): Detail of the 18q21.2- q23 gene region showing the segment deletion of approximately 26.61-Mb from sample 3 (Red Line). (D): FISH with the 18 centromere/DXZ1 probe (Aqua) and BCL2/18q21.33 region probe. The absence of the red signal on chromosome 18 homologue confirm the deletion of the critical region identified by array-CGH. (E): FISH with with the 18 centromere/DXZ1 (Aqua Signal), subtelomere/18p11.32 probe (Green Signal) and subtelomere 18q/18q23 probe (Red Signal) indicates the occurrence of an inversion of the long arm of chromosome 18 (Localized on the Right Side). (D;E): Probe from Cytocell, Cytocell Ltd., Cambridge, UK. BCL2: B-Cell CLL/Lymphoma 2

The patient with del18q21.2-q23 was referred to the clinical genetics department at age of 3 years. Dysmorphic features were noted (Table1), including microcephaly, cerebral hypoplasia, bitemporal narrowing, epicanthus, long thumbs, sacral dimple and hypotonia. She had psychomotor and speech delay and stereotyped movements. Together, the clinical and cytogenetic evaluation suggested the patient's has the Pitt-Hopkins syndrome [29]. Pitt-Hopkins syndrome (PTHS, MIM #610954) is caused by haploinsufficiency of the TCF4 gene at 18q21.2 due to deletions, splice-site and, less frequently, missense mutations which occur de novo.

PTHS is characterized by severe intellectual disability, typical facial features and tendency to epilepsy, panting-and-holding breathing anomaly, stereotypic movements, constipation, and high-grade myopia. Growth is normal or only mildly retarded, but half of the patients have postnatal microcephaly. PTHS is not extremely rare among patients with severe intellectual disability. During a study period of 17 months, Rosenfeld [30] screened 13,186 samples from intellectually disabled individuals by array-CGH, and they found 7 persons carrying a deletion which included the whole of or a part of the TCF4 gene. From these results, it was estimated that the frequency of PTHS caused by microdeletion is 1/34,000-1 /41,000 in Washington, USA. The true prevalence of PTHS should be higher, as many cases are caused by point mutations.

At present, there is no evident phenotype-genotype correlation as regards the intragenic deletions or point mutations of the TCF4 gene [31]. In the the 18q deletion patients, hemizygosity for TCF4 appears to confer a major impact with regard to motor and cognitive development: in one study, children with larger regions of hemizygosity as the one reported here, including as several contiguous genes, were not more developmentally delayed than children with hemizygosity for the TCF4 gene alone [32]. In contrast, patients, with large deletions but haplosufficient for TCF4 had milder symptoms and longer survival. Hemizygosity for TCF4 confers a significant impact primarily with regard to cognitive and motor development, resulting in a very different prognosis for individuals hemizygous for TCF4 when compared to individuals hemizygous for other regions of distal 18q segment.

With regard to cytogenetic and molecular examinations of sample 4, the duplication was initially identified through G-banding. The karyotype was interpreted as 46, XY, dup(8) (q24). The precise characterization of the deletion using oligonucleotide aCGH showed a loss of genomic material corresponding to an interstitial duplication in the long arm of chromosome 8, segment q24.13-q24.3, of approximately 17.18 Mb (UCSC Genome Browser on Human Dec. 2013 (GRCh38/hg38). Arr 8q24.13-q24.3 (125,385,074 -142,496,610) ×3 (Figure 4). Furthermore, FISH with MYC/8q24.21 probe and GSDMC/8q24.1 probe confirmed

the duplication identified by array-CGH revealing also the occurrence of an inversion of the long arm of chromosome 8 (Figure 4C). G- banding and FISH analyses of the paternal chromosomes revealed normal results.

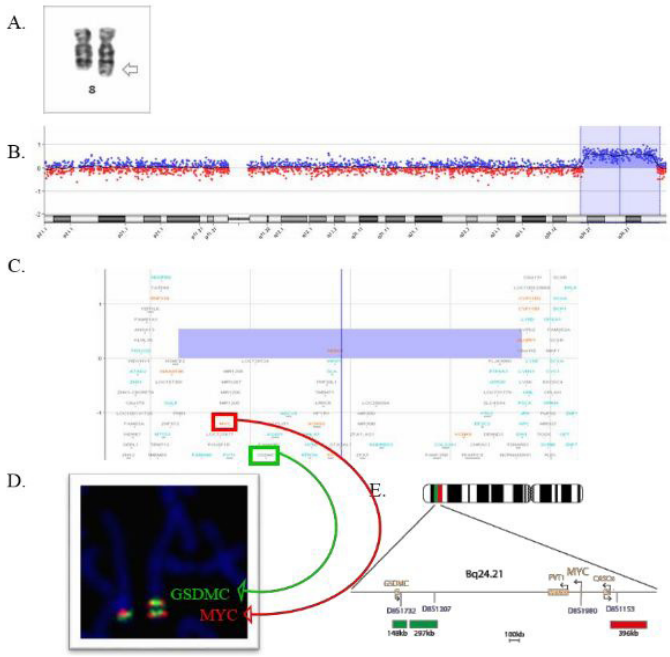

Figure 4(A-D): (A): Chromosome banding shows an additional segment on the long arm of chromosome 8q24. **(B):** Array-CGH ratio profiles of chromosome 8q24.13-q24.3. The test/reference ratio data for the chromosome 8 is shown. A copy number gain shifts the ratio upward (approximately +1x). **(C):** Detail of the 8q24.13-q24.3 gene region showing the segment duplication of approximately 14.75-Mb from sample 4 (Blue Line). FISH with MYC/8q24.21 probe (Red Signal) and GSDMC/8q24.1 probe (Green Signal). **(D):** Chromosome scheme showing the respective localization of the probes utilized in the FISH analysis. Probes from Cytocell, Cytocell Ltd., Cambridge, UK; GSDMC (Gasdermin C); MYC (V-Myc Avian Myelocytomatosis Viral Oncogene Homolog).

However, the maternal karyotype showed the same chromosome banding pattern of the patient, revealing the same duplication of the chromosome 8 as the child (Data Not Shown). FISH analysis using probes MYC/8q24.21 and GSDMC/8q24.1 showed an inverted duplication on the abnormal 8 chromosome as well. When the duplicated segment of breakpoints falls within the coding sequence,and duplication is in tandem, the duplicated material is in direct orientation to the original copy. In these cases, there is still likely to 2 reading frames intact and open to the affected gene, with a third reading frame interrupted. This is less likely to result in clinical consequence. However, if the duplicated material is inverted, there must be only one intact open reading frame and increasing the probability of a clinical consequence due to haploinsufficiency of the disrupted gene [33].

Duplications detected by genomic microarray present unique considerations, such as location, orientation, and breakpoints of the additional copy of genomic material (Figure 4). Most interstitial duplications are tandem and in direct orientation in relation to the original locus. However, the location of the additional material may itself require consideration. FISH with a probe in the duplicated region can often show the genomic location of the additional material, and support either a tandem duplication or inversion duplication event, as we have seen in sample 4 (Figure 4). The only inverted duplications are those that are part of more complex rearrangements, including insertional translocations and inverted duplications adjacent to terminal deletions [34].

Interstitial duplications are often inherited from parents. In our study, the interstitial duplication characterized is maternal in origin. The mother had medical history of cleft lip and palate, not observed in his son. Thus, predicting outcomes for future pregnancies complicated by incomplete penetrance and variable expressivity that can occur in individuals of the same family, complicating the genetic counseling. The highly variable nature of the genome means that care must be taken in assigning pathogenicity to inherited chromosomal rearrangements. Regarding the cleft lip and palate described in the mother, we shall consider that approximately 30% of cleft lip and palate cases and 50% of cleft palate cases are recognized as components of MCA syndromes [35]. However, both genetic and environmental factors are known to contribute to the occurrence of cleft lip and palate, complicating the elucidation of the causative mechanisms. Considerable efforts have been made in seeking candidate gene(s) for non- syndromic clefts through array-CGH, showing that it is an effective method for isolating candidate loci [36,37].

Currently, the molecular detection of structural chromosome rearrangements in individuals with MCA as well as in affected parents is prone to diagnostic pitfalls due to difficulties in interpretation, as already revised in other structural variant studies [11]. Most chromosomal abnormalities have clinical effects; however, some genomic rearrangements often display variable expressivity and penetrance [38,39]. Using combined analytic tools is necessary to delineate the entire range of variation that is associated with a particular structural rearrangement in an individual personal genome.

To clinically manage situation with inherited chromosomal rearrangements it is essential to have the most accurate and up-to-date information on the clinical significance of known genomic deletions and duplications, pathogenic mutations, polymorphisms and non-genetic factors that may lead to a disease trait. Further consultations at clinical genetics and extended analysis of family members may be necessary to provide accurate clinical examinations, genetic counseling and calculation of the recurrence risk of chromosome rearrangements.

We report here two interstitial deletions, one inherited (del3q11.2q13.31) and one *de novo* (del6p24.3p22.3) cytogenetically visible. The introduction of array CGH has enabled to detect previously unrecognized imbalances. Array-CGH can define precisely the size of a larger or small imbalance allowing the presence of LCRs at the breakpoints to be investigated. This makes possible to distinguish rearrangements formed by NAHR from rearrangements formed by other mechanisms. Many factors contribute to the formation of genomic structural rearrangements [40]. The formation of recurrent microdeletion/ duplication syndromes is mediated by Non-Allelic Homologous Recombination (NAHR) mediated by Low Copy Repeats (LCRs), predominantly during meiosis [16]. We assumed that an imbalance have been mediated by NAHR if paralogous LCRs spanned all or a large proportion of both breakpoint intervals of a chromosome rearrangement, while an imbalance was assumed to have arisen by a mechanism other than the NAHR if LCRs were absent from breakpoint intervals. For the two simple larger imbalances described here, an LCR was present at only one breakpoint interval and these were also assumed to have arisen by a mechanism other than the NAHR. This is in agreement with the formation of non-recurrent chromosome imbalances, which appears to be much more heterogeneous.

Patients 3 and 4 presented inversions associated with segmental deletion and duplication, respectively. In both cases, genomic imbalances could not be diagnosed neither by classical cytogenetic studies nor by array-CGH. The event of inversion could be identified only after Fluorescence *In Situ* Hybridization (FISH) analysis using locus- specific probes within for critical segments (Figures 3C and 4C). Genomic microarray can identify copy number changes, as deletions and duplications, but it does not show further structural rearrangements, as inversions or balanced translocations. In some chromosomal structural rearrangements, as the inversion/deletion and inversion/duplication observed in our study, only FISH analysis can further characterize an imbalance detected.

It is also possible that multiple chromosome alterations may exist in a single individual. The cytogenetic recognition of 1 alteration may satisfy the inquiry for the etiology of the patient's phenotype; however, a higher resolution analysis may reveal an unexpected additional alteration that has clinical significance or another relevant structural rearrangement. Examples include the initial interpretation of a direct terminal deletion, as firstly assumed in patient 3 (del18q21.2-q23), or a direct duplication, as previously assumed in patient 4 (dup824.13-q24.3), in who FISH analysis shows an unsuspected inversion. In both events of inversion/deletion and inversion/duplication reported here, it could be suggested that the lack of homology across the inversion region between heterozygous chromatids in meiosis may lead to the formation of an "Asynaptic Bubble" that renders the region unstable and prone to additional rearrangements [41].

The clinical cases presented here could not be determined only by clinical examination. In general, phenotypic characteristics of well-defined deletion/duplication chromosomal rearrangements associated with MCA presenting or not intellectual disabilities are often clinically detected before the causal structural rearrangements are identified [42]. However, the clinical evaluation of individuals with such syndromes continues to be a challenge for clinicians and requires a high degree of experience and expertise. Although some diagnostic steps are highly standardized (for example, database searches, clinical utility gene cards, and standard clinical scores), others are not suitable for standardization.

Moreover, the diagnosis of interstitial deletions and duplications as reported in our study, using only clinical assessment, may be difficult due to the great symptoms variability, especially in relation to the size of the genomic imbalance and the expertise of the clinician. In contrast to single gene disorders, contiguous gene deletions and duplications, and especially those resulting in developmental delays, intellectual disabilities or congenital developmental abnormalities, are caused by structural chromosomal rearrangements that encompass several genes; generally, at least two of these genes are dosage-sensitive but functionally unrelated. There are also some features that do not become distinct until a certain age, at which time a particular behavior or clinical manifestation presents. Because of the growing number of recognized genetic syndromes and chromosome abnormalities, and because of the overlapping clinical characteristics of carriers of segmental deletions and duplications, it is becoming increasingly difficult to use only the clinical examination to exactly determine the syndrome that affects an individual who carry a chromosomal structural rearrangement.

Although considerable improvements have recently been made in mapping structural rearrangements and interpreting their functional impact, numerous important challenges remain. The characterization of the critical region related to specific congenital defects depends also on the quality of the phenotyping, the local practice and the availability of funding. For example, in this study, we should consider the prior subjects' limited access to appropriate cytogenomic studies and care, which is the case in most regions in low- and middle-income countries [43].

Conclusions

In conclusion, our study has shown that combined cytogenomic methods can be successfully used to characterize genomic imbalances in individuals with interstitial deletions and duplications associated with malformation syndromes in the clinical practice. The use of retrospective or prospective cytogenomic analysis of chromosomal structural rearrangements as a diagnostic tool would benefit families by providing a more accurate diagnosis and would affect overall disease management in a significant number of cases. Indeed, it is important to report cytogenetic data when characterizing structural rearrangements because some of the data may represent recurrent genomic imbalances that could be associated with a specific syndrome not yet classified. Reports of subjects with similar genomic imbalances, as well as clinical findings, may also lead to the identification of newly recognized genomic disorders or candidate genes. Furthermore, the results of such studies emphasize the growing importance of the use of a combination of technologies for both the identification and characterization of segmental deletions and duplications, thereby increasing the understanding on the dynamic nature of chromosome structure and its relationship with genomic diseases. The technology used to study genomic structural rearrangements has also rapidly expanded, and the number of genomic rearrangements in the human genome are likely unlimited. Therefore, comprehensive characterization of structural rearrangements through various approaches represents a major challenge to understand how chromosomes imbalances arise.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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