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**Técnicas de análise genômica permitem estabelecer o
diagnóstico etiológico de crianças com baixa estatura de causa
desconhecida**

Tese apresentada à Faculdade de Medicina da
Universidade de São Paulo para obtenção de
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Programa de Endocrinologia

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Jorge
Coorientadora: Prof. Dra. Alessandra Christianne
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in memoriam

Exemplos de força, coragem, integridade e determinação

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RESUMO

Homma TK. *Técnicas de análise genômica permitem estabelecer o diagnóstico etiológico de crianças com baixa estatura de causa desconhecida*. [tese]. São Paulo: Faculdade de Medicina, Universidade de São Paulo; 2019.

INTRODUÇÃO: Crianças com baixa estatura constituem um grupo heterogêneo. Em uma parcela dos casos, o mecanismo envolvido nesse processo decorre de alterações genéticas. **OBJETIVO:** Realizar uma investigação clínica e genético-molecular de um grupo de pacientes com baixa estatura de causa desconhecida. **MÉTODOS:** Seleccionamos crianças com baixa estatura persistente (escore-Z de altura ≤ -2 para idade e sexo) de causa desconhecida para avaliação genômica. O estudo foi dividido em 2 etapas: 1ª etapa – avaliação de 229 pacientes com baixa estatura síndrômica [baixa estatura associada a outros achados dismórficos (atraso de desenvolvimento neuropsicomotor e/ou déficit intelectual, presença de dismorfismos faciais e/ou outras malformações)] por cariótipo molecular (aCGH/SNPa); 2ª etapa: avaliação de 99 crianças com baixa estatura persistente, nascidas pequenas para idade gestacional (PIG - escore-Z de peso e/ou comprimento ao nascer ≤ -2 para idade gestacional) e classificação de acordo com a presença ou ausência de dismorfismos associados. Essas crianças foram divididas em dois grupos: baixa estatura síndrômica (n=44) e baixa estatura isolada (n=55). Pacientes com baixa estatura síndrômica foram avaliados por sequenciamento exômico (WES). Pacientes com baixa estatura isolada foram avaliados através de painel gênico (n = 39) ou WES (n = 16). **RESULTADOS:** 1ª etapa: 32 (14%) pacientes com baixa estatura síndrômica apresentaram variações no número de cópias (CNVs) patogênicas ou possivelmente patogênicas. Sete delas são recorrentes em outros estudos e são responsáveis por cerca de 40% de todas as CNVs patogênicas/possivelmente patogênicas encontradas em pacientes com baixa estatura de causa desconhecida. 2ª fase: Dentre os 99 pacientes avaliados com baixa estatura nascidos PIG, foram encontradas 23 variantes patogênicas/possivelmente patogênicas em genes já associados a distúrbios de crescimento. Quinze (34%) nos pacientes com baixa estatura síndrômica, em genes relacionados a processos celulares fundamentais, vias de reparo de DNA e vias intracelulares; e oito (15%) em pacientes com baixa estatura isolada, em genes associados à cartilagem de crescimento e a via RAS/MAPK. **CONCLUSÃO:** A heterogeneidade dos pacientes com baixa estatura dificulta o diagnóstico clínico. As novas abordagens genômicas permitem estabelecer o diagnóstico etiológico de crianças com baixa estatura de causa desconhecida.

Descritores: transtornos do crescimento; insuficiência de crescimento; retardo do crescimento fetal; genética; sequenciamento completo do exoma; hibridização genômica comparativa.

ABSTRACT

Homma TK. *Genomic analysis techniques allow the establishment of etiological diagnosis in short stature children of unknown cause*. [thesis]. São Paulo: “Faculdade de Medicina, Universidade de São Paulo”; 2019.

BACKGROUND: Patients born small for gestational age (SGA) are a heterogeneous group, and in several cases, it is due to genetic processes. **AIM:** To perform a clinical and genetic-molecular investigation of short stature patients of unknown cause. **METHODS:** We selected short stature children (height ≤ -2 SDS for age and sex) of unknown cause for genomic evaluation. The study had two stages: 1st stage - 229 syndromic short stature patients (patients with short stature and dysmorphic features, developmental delay, and/or intellectual disability) were evaluated by molecular karyotype (aCGH/SNPa); 2nd stage: We selected 99 short stature children born SGA (birth weight and/or length ≤ -2 SDS for gestational age). They were classified according to the presence or absence of dysmorphic features into two groups: syndromic short stature (n=44) and isolated short stature (n=55). Patients with syndromic short stature were evaluated by whole exome sequencing (WES), and patients with isolated short stature were evaluated through a target panel sequencing (n=39) or WES (n=16). **RESULTS:** 1st stage: 32 (14%) syndromic short stature patients had pathogenic or probably pathogenic copy number variations (CNVs). We observed seven recurrent CNVs that are responsible for about 40% of all pathogenic/probably pathogenic genomic imbalances found in short stature patients of unknown cause. 2nd stage: Of the 99 patients evaluated, 23 pathogenic/likely pathogenic variants were found in genes already associated with growth disorders. Fifteen (34%) syndromic short stature patients had pathogenic variants in genes related to fundamental cellular processes, DNA repair and intracellular pathways; and eight (15%) isolated short stature patients had pathogenic variants in genes associated with growth plate development and the RAS/MAPK pathway. **CONCLUSION:** The heterogeneity of short stature makes the clinical diagnosis difficult. The new genomic approaches are effective to diagnose a larger number of undiagnosed patients.

Descriptors: growth disorders; failure to thrive; fetal growth retardation; genetics; whole exome sequencing; comparative genomic hybridization.

INTRODUÇÃO

Nos últimos anos, o desenvolvimento da genética e biologia molecular forneceu uma grande quantidade de dados e informações que reforçaram o conceito de que a maioria das características e doenças humanas tem algum componente genético (1). E a determinação desse componente proporcionaria oportunidades notáveis para medicina clínica através de uma melhor compreensão da patogênese, diagnóstico e opções terapêuticas (1).

Em relação ao crescimento, acredita-se que a altura seja uma característica hereditária complexa, até 90% da variação de estatura seria decorrente de fatores genéticos (2). Estudos de associação genômica já identificaram 3290 locus independentes associados à altura, demonstrando uma natureza altamente poligênica (3). Ainda assim, os loci identificados explicariam cerca de 24,6% da variabilidade na altura adulta, indicando que outras alterações com menor frequência e/ou variações no número de cópias poderiam estar relacionadas à esse processo (3).

Dessa forma, acredita-se que à variabilidade de altura em indivíduos saudáveis poderia ser explicada por combinação de diversos polimorfismos (4, 5), e crianças com baixa estaturas mais graves (escore- $Z \leq -3$) e/ou presença de características dismórficas associadas, como malformações, atraso de desenvolvimento e/ou déficit intelectual, ou histórico familiar de baixa estatura teriam maior probabilidade de uma alteração genética decorrente de uma mutação monogênica ou variações no número de cópias de genes contíguos (6, 7).

Considerando a heterogeneidade dos pacientes com baixa estatura, o estabelecimento preciso desse diagnóstico é importante para o prognóstico, seguimento e aconselhamento genético (8-10). Entretanto, o reconhecimento clínico das causas genéticas muitas vezes não é tão fácil de ser realizado. Existem aproximadamente cerca de 2000 condições genéticas onde a baixa estatura é uma das características cardinais descritas e cerca de 300 novos fenótipos são adicionados ao OMIM® a cada ano (11-13). Os casos típicos e/ou com maior frequência, normalmente, são facilmente reconhecidos. Porém, muitas condições apresentam espectro clínico variável e, em outros casos, as mesmas características clínicas podem se sobrepor entre os diferentes diagnósticos, tornando a identificação da doença mais difícil. Além disto, várias condições são

extremamente raras dificultando o reconhecimento mesmo por grupos especializados (6, 10, 13-16).

Dessa forma, diante de um paciente com baixa estatura, sindrômica ou isolada, muitas vezes, o pediatra e/ou endocrinologista se provém de uma série de exames laboratoriais, de imagem, avaliação por outros especialistas para que o diagnóstico etiológico da baixa estatura seja estabelecido. Entretanto, muitas vezes os exames são normais e/ou não justificam o quadro clínico, e o paciente se mantém sem um diagnóstico definitivo (17-19).

Nesse contexto, desde 1997, nosso grupo vem buscando estudar pacientes com baixa estatura sem etiologia identificada através de estudos genéticos. Inicialmente, buscou-se avaliar genes específicos já associados à baixa estatura através do sequenciamento pelo método de Sanger (20-23) e *multiplex ligation-dependent probe amplification* (MLPA) (24, 25). Entretanto, apesar da possibilidade de confirmação diagnóstica em vários casos, a avaliação gene-a-gene se mostrou laboriosa e demorada.

Em 2014, começamos a realizar estudos com abordagem genômica, através da avaliação do número de cópias cromossômicas (CNVs) por hibridização genômica comparativa por *microarray* (aCGH) e por avaliação de polimorfismos em nucleotídeos únicos (SNPa) (26, 27). As análises dos CNVs permitiram identificar a base genética de várias doenças, tornando possível a detecção e o mapeamento de todo o genoma em busca de deleções e duplicações submicroscópicas com maior sensibilidade e resolução, além de revelar novos genes e/ou loci relacionados à condição estudada (28). Entretanto, muitos pacientes permaneceram ainda sem diagnóstico.

Nos últimos anos, ganhou destaque as tecnologias de sequenciamento em larga escala, especialmente o sequenciamento exômico (WES), que permitiu o estudo das regiões de DNA contendo os exons codificadores de proteínas, que apesar de compor apenas 1% a 2% do genoma, contém cerca de 85% de todas as mutações causadoras de doenças (29). Alguns estudos tem mostrado que essa tecnologia tem permitido o estabelecimento do diagnóstico de diversas condições monogênicas, de forma mais rápida e muitas vezes com menor custo (6, 30-33).

Portanto, a proposta do estudo foi investigar clinicamente e através de exames genéticos (cariótipo molecular e sequenciamento paralelo em larga escala) grupos de pacientes com baixa estatura isolada e sindrômica sem etiologia identificada, na tentativa de estabelecer a causa genética.

OBJETIVO GERAL

Identificar causas genéticas de baixa estatura através de investigação clínica e molecular.

OBJETIVOS ESPECÍFICOS

- Avaliar a presença de variações no número de cópias patogênicas em crianças com distúrbio de crescimento e características sindrômicas através da metodologia de cariótipo molecular e analisar presença de CNVs recorrentes associadas ao fenótipo.
- Estabelecer o diagnóstico genético de crianças sindrômicas com baixa estatura de início pré-natal de causa não identificada utilizando sequenciamento exômico.
- Avaliar a frequência de causas genéticas em crianças não sindrômicas com baixa estatura de início pré-natal utilizando sequenciamento paralelo em larga escala.

RESULTADOS

Dada a complexidade do assunto, o estudo permitiu a confecção de quatro artigos originais que serão apresentados na forma em que foram preparados para publicação. Os artigos contaram com a colaboração fundamental de outros pesquisadores durante a condução do estudo, como mostrado na autoria dos manuscritos. A descrição específica dos métodos utilizados e resultados de cada um dos estudos que compõe a tese está no corpo dos textos apresentados.

CAPÍTULO 1 - *Recurrent copy number variants associated with syndromic short stature of unknown cause*

O estudo da variação do número de cópias (CNVs), através da metodologia de cariótipo molecular, como uma ferramenta de diagnóstico clínico, levou a detecção de desequilíbrios cromossômicos causadores de uma variedade de síndromes genéticas, que englobam desde doenças neurológicas, como atraso de desenvolvimento e epilepsia, até obesidade e malformações cardíacas (34-37). A utilização dessa tecnologia, além de facilitar a identificação de novas síndromes de microdeleção e microduplicação, permitiu a identificação de genes associados à diversas doenças (38, 39).

Na avaliação de crianças com baixa estatura, as CNVs parecem desempenhar um papel importante. Estudos tem descrito uma frequência de 10-16% de CNVs patogênicas ou possivelmente patogênicas dentre crianças com baixa estatura, especialmente dentre aqueles com atraso no desenvolvimento e/ou déficit intelectual e malformações congênitas (26, 40-42).

Enquanto testes mais direcionados para genes específicos de baixa estatura ou síndromes genéticas são indicados para alguns pacientes com base no fenótipo clínico, o cariótipo molecular é uma excelente ferramenta diagnóstica para baixa estatura síndrômica, permitindo identificar causas genéticas entre pacientes para os quais o quadro clínico não sugere uma avaliação diagnóstica mais focada ou pacientes para os quais o fenótipo completo ainda não se manifestou no momento (1, 10).

O objetivo desse estudo foi analisar uma grande coorte de crianças com baixa estatura associada a características dismórficas sem etiologia conhecida em busca de CNVs. Além disso, realizou-se pesquisa na literatura de outros estudos que utilizaram a metodologia de cariótipo molecular em pacientes com baixa estatura afim de identificar CNVs recorrentes.

O artigo decorrente desse estudo, foi publicado na revista *Horm Res Paediatr.* 2018;89(1):13-21. doi: 10.1159/000481777, onde ganhou destaque, sendo escolhido pelo editor para ser a capa da edição e recebeu *free access*.

HORMONE RESEARCH IN PÆDIATRICS

From Developmental Endocrinology to Clinical Research

The importance of copy number variants in the pathogenesis of short stature

(see paper by Homma et al., pp. 13–21)

Recurrent Copy Number Variants Associated with Syndromic Short Stature of Unknown Cause

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Keywords

Short stature · Chromosomal microarray · Copy number variants · Recurrent copy number variants · Array-based comparative genomic hybridization · Single nucleotide polymorphism array

Abstract

Background/Aims: Genetic imbalances are responsible for many cases of short stature of unknown etiology. This study aims to identify recurrent pathogenic copy number variants (CNVs) in patients with syndromic short stature of unknown cause. **Methods:** We selected 229 children with short stature and dysmorphic features, developmental delay, and/or intellectual disability, but without a recognized syndrome. All

patients were evaluated by chromosomal microarray (array-based comparative genomic hybridization/single nucleotide polymorphism array). Additionally, we searched databases and previous studies to recover recurrent pathogenic CNVs associated with short stature. **Results:** We identified 32 pathogenic/probably pathogenic CNVs in 229 patients. By reviewing the literature, we selected 4 previous studies which evaluated CNVs in cohorts of patients with short stature. Taken together, there were 671 patients with short stature of unknown cause evaluated by chromosomal microarray. Pathogenic/probably pathogenic CNVs were identified in 87 patients (13%). Seven recurrent CNVs, 22q11.21, 15q26, 1p36.33, Xp22.33, 17p13.3, 1q21.1, 2q24.2, were observed. They are responsible for about 40% of all pathogenic/probably pathogenic genomic imbalances found in short stature

patients of unknown cause. **Conclusion:** CNVs seem to play a significant role in patients with short stature. Chromosomal microarray should be used as a diagnostic tool for evaluation of growth disorders, especially for syndromic short stature of unknown cause.

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Introduction

Short stature is a common reason for children to be evaluated by a specialist. The etiology of short stature is heterogeneous and, usually, the diagnostic approach to this condition is based on clinical evaluation complemented by laboratory and radiological exams [1–3]. Even though many short stature cases remain without a specific diagnosis, it is estimated that a large fraction of this short stature of unknown etiology has a genetic cause. The genetic evaluation of short stature is important not only for diagnosis, but also to provide additional information to the patients and their families regarding natural history, prognosis, available treatment, and precise genetic counseling [4]. For decades, candidate gene approaches have been applied in the molecular-genetic investigation of children with growth disorders. However, the genetic heterogeneity in short stature conditions, the rarity of some diseases, and the considerable variability in phenotypes may impair an etiological diagnosis based only on this approach [5, 6]. Recently, with the advent of new technologies, a genomic approach arose as an important strategy for genetic investigation and to establish the etiology of growth disorders [5, 6].

In this scenario, analyses of chromosomal copy number variants (CNVs) have provided an opportunity to identify the genetic basis of several human diseases. Array-based genomic copy number analyses, including array-based comparative genomic hybridization (aCGH) and single nucleotide polymorphism arrays (SNPa), allow detecting and mapping submicroscopic deletions and duplications with higher sensitivity and resolution [7]. Recent studies have identified pathogenic or probably pathogenic CNVs in 10–16% of children with short stature of unknown cause [8–11], usually in patients with additional malformations and/or neurodevelopmental disorders. Results have shown that rare CNVs contribute as significant genetic causes to short stature in these patients and also reveal novel potential candidate related-genes and/or loci [8–12].

Based on these observations, the objective of this study was to determine the frequency of rare CNVs and to de-

scribe novel CNVs in a large cohort of patients with syndromic short stature of unknown cause. We also reviewed the scientific literature regarding CNVs in short stature, to identify recurrent pathogenic or probably pathogenic CNVs associated with this condition.

Materials and Methods

Subjects

The Local Ethics Committee approved this study, and the patients and/or their guardians gave written informed consent. The cohort consisted of 229 patients from a pediatric endocrinology outpatient clinic ($n = 62$) and from a University Genetic Center ($n = 167$) referred for molecular genetic investigation. The patients from the pediatric endocrinology outpatient clinic were included based on the following criteria: short stature at the age of 2 years or above (height standard deviation score ≤ 2) and presence of dysmorphic features, developmental delay, and/or intellectual disability, but without a recognized syndrome. The patients from the University Genetic Center were referred for chromosome microarray analysis for presenting intellectual disability or developmental delay; included among them in this study were those patients with short stature (standard deviation score ≤ 2), many of them presenting additional clinical signs. All patients had normal G-banded karyotyping.

Methods

Genomic DNA was extracted from peripheral blood leukocytes of all patients using standard procedures. aCGH ($n = 71$) or SNP array ($n = 168$) were performed according to availability. All experiments were conducted according to the standard protocol of the manufacturer or previously published data [10, 13]. aCGH was performed in a whole-genome 180 K platform (Agilent Technologies, Inc., Santa Clara, CA, USA). Microarray-scanned images were processed using the Software Genomic Workbench (Agilent Technologies, Inc.). The SNP array used was the CytoSNP-850K BeadChip (Illumina, USA) or CytoScan HD array (Affymetrix Inc., Santa Clara, CA, USA). Microarray-scanned images were processed using Bluefuse Multisoftware v4.1 (Bluegenome, UK) or Affymetrix® Chromosome Analysis Software Suite (ChAS) v.3.0.0.42 (Affymetrix). The parameters used to call a duplication or a deletion were $\log_2$ ratio intensities of a given genomic segment >0.3 or <-0.3 , respectively, and encompassing at least 3 probes. CNVs considered common were excluded, based on the Database of Genomic Variants [14] and an independent control cohort of 400 healthy individuals.

We collected genomic coordinates, size, genes encompassed and, when available, inheritance of the identified CNVs. The assessment of CNV pathogenicity was made by considering the criteria based on the Consensus Statement of Chromosomal Microarray [7] and the guidelines of the American College of Medical Genetics [15], consistent with clinical phenotype, and updated information on known short stature syndromes and growth impairment-related genes. Briefly, we classified the CNVs as follows: (1) pathogenic: CNVs that overlap with genomic coordinates for a known genomic-imbalance syndrome and CNVs ≥ 3 Mb and (2) probably pathogenic: CNVs between 1 and 3 Mb or CNVs ≥ 300 kb carrying Online Mendelian Inheritance in Man (OMIM) mor-

bid genes de novo or inherited from a parent with a similar phenotype. A total of 16 relatives of 8 patients were analyzed to investigate probably pathogenicity and inheritance of CNVs. Parents of patients who had clear pathogenic and large CNVs (more than 3 Mb) were not tested, since the classification of pathogenicity was not dependent on the inheritance in these cases.

Literature Search Strategy

We searched the literature for studies evaluating CNVs in cohorts of patients with short stature up to April 2017 using the following criteria: (1) published in English, (2) investigation of patients with short stature of unknown cause, and (3) contained enough information about the results and methodology used in the study. We excluded non-original studies, case reports, specific disease investigation, custom genome-wide microarrays, and articles not published in English. We used the following search terms: (short stature OR growth impairment OR growth restriction OR growth retardation OR height OR dwarfism OR dwarf) AND (copy number variants OR array comparative genomic hybridization OR aCGH OR single nucleotide polymorphism array OR SNP OR chromosomal microarray OR molecular karyotyping). Furthermore, we manually searched the reference lists of every primary study for additional information.

Data of CNVs and their genomic positions were collected from all the selected studies. The recurrent loci were selected, and the genomic positions were analyzed by DECIPHER [16]. The protein coding genes situated in CNVs loci were analyzed by VarElect NGS Phenotyper Program [17]. We used the following phenotype terms for prioritization of genes: “short stature” OR “growth impairment” OR height OR dwarfism OR dwarf OR “growth restriction” OR “growth retardation.” We selected the first 5 genes directly related to the phenotype. The assessment of gene function and the assessment of overlapping with other genomic disorders were performed using the OMIM and the PubMed databases.

Results

Analysis of 229 Patients with Short Stature of Unknown Cause

Among 229 patients with short stature studied by chromosomal microarray analysis, we observed 77 rare CNVs in 73 patients (1–4 per patient). We classified 25 CNVs as pathogenic and 7 as probably pathogenic. Moreover, 2 patients have maternal uniparental disomies. Pathogenic/probably pathogenic CNVs presented the following main characteristics: sizes ranged from 0.5 to 6.7 Mb (84.4% were over 1 Mb); 26 were deletions while 6 were duplications. Nineteen CNVs overlapped with genomic coordinates for a known microdeletion/microduplication syndrome already associated with short stature (Table 1).

The most recurrent phenotypes associated with short stature among patients with pathogenic/probably pathogenic CNVs were developmental delay and/or intellectual

disability ($n = 29$, 85.3%), dysmorphic facial features ($n = 23$, 67.6%), microcephaly ($n = 9$, 26.5%), cardiac congenital anomalies ($n = 3$, 8.8%), and cryptorchidism ($n = 2$, 5.9%). Fifty patients presented with minor anomalies, such as strabismus, pectus excavatum, clinodactyly, sacral dimple, hypothyroidism, and 1 patient had renal malformation. The online supplementary Table (for all online suppl. material, see www.karger.com/doi/10.1159/000481777) summarizes clinical and genetic features of all children with detected pathogenic/probably pathogenic CNVs.

Analysis of Recurrent CNVs Associated with Risk for Short Stature

Reviewing the literature, we found 6,029 studies which used chromosome microarray technologies. Of these 258 included patients with growth disorders or short stature. We performed a manual curation based on abstracts. They were analyzed according to our including and excluding criteria previously cited. This strategy resulted in a final selection of 4 eligible studies to be included in the CNV recurrence analysis [8–11] (Table 2). These studies and the present one reported a total of 671 patients with short stature of unknown cause evaluated by chromosome microarray technologies (SNPa or aCGH). Pathogenic/probably pathogenic CNVs were identified in 87 patients (13%; 95% confidence interval of 10.4–15.5%). We identified 7 recurrent CNVs in short stature (Table 3). Four of these recurrent CNVs were described in multiple studies (22q11, 15q26, Xp22.33, 1p36.33, and disomy in chromosome 14) and 2 recurrent CNVs were found in a single study and involved unrelated patients (1q21.1 and 17p13.3).

Discussion

In the past few years, since the genomic approaches have been advancing, an increasing number of causative genetic alterations have been identified in many diseases. It has been demonstrated that CNVs (i.e., deletions or duplications of chromosomal segments) have a strong relationship with genome variability and contiguous gene syndrome and might be responsible for several conditions associated with short stature [18]. In 2009, the American College of Medical Genetics published the first practice guideline for genetic evaluation of short stature, which includes the recommendation for CNV investigation in patients with proportional short stature and other physical or developmental defects with an unrecognized syndrome [4].

Table 1. Description of the genomic imbalances classified as pathogenic/probably pathogenic

Cytogenetic position	Genomic position (hg 19) ¹	Size, Mb	Gain/loss	Pathogenicity	Affected genes, <i>n</i>	Protein coding/OMIM genes, <i>n</i>	MD syndrome ²
1p36.33	chr1:1018337-2188572	1.1	Loss	Pathogenic	49	43/13	1p36 microdeletion syndrome
1p36.33p36.32	chr1:82154-2366316	2.2	Loss	Pathogenic	80	58/35	1p36 microdeletion syndrome
2p15p16.1	chr2:60910033-64133562	3.2	Loss	Pathogenic	37	20/17	2p15-16.1 microdeletion syndrome
2q24.1q24.2	chr2:156761199-163169595	6.4	Loss	Pathogenic	54	28/24	
2q24.2q24.3	chr2:161229701-167823275	6.6	Loss	Pathogenic	44	24/22	
2q24.3q31.1	chr2:169465503-170164641	0.7	Gain	Pathogenic	9	7/7	Chr 2q31.1 duplication syndrome
2q37.2q37.3	chr2:236798070-242717216	6.7	Loss	Pathogenic	80	58/42	
4p16.3p16.2	chr4:49450-5516502	5.5	Loss	Pathogenic	93	62/49	Wolf-Hirschhorn syndrome
7q36.1q36.2	chr7:150412317-152604342	2.2	Loss	Probably pathogenic	47	31/28	
8q23.3q24.13	chr8:117628200-122637676	5.0	Loss	Pathogenic	32	21/18	
9q22.2q22.33	chr9:93184497-99876878	6.7	Gain	Pathogenic	89	46/30	
10q22.2	chr10:76540987-77666682	1.1	Loss	Probably pathogenic	15	8/6	
10q26.3	chr10:131188376-135253581	4.1	Loss	Pathogenic	45	28/19	
12q14.2q15	chr12:64082220-69757429	5.7	Loss	Pathogenic	64	32/29	12q14 microdeletion syndrome
12q24.23q24.31	chr12:120308438-122331128	2.2	Gain	Probably pathogenic	57	38/34	
14q24.3q32.33	chr14:73972535-107287663	33.3	UPD	Pathogenic ²	620	224/168	Temple syndrome
14q31.3q32.33	chr14:88045320-107285437	19.2	UPD	Pathogenic ²	505	148/114	Temple syndrome
15q11.1q11.2	chr15:20212798-23226254	3.0	Loss	Pathogenic	62	10/5	Spastic paraplegia 6
15q11.2q13.1	chr15:23656946-29006093	5.3	Loss	Pathogenic	117	15/12	Chr 15q11.2 deletion syndrome
15q13.3	chr15:32018731-32513233	0.5	Gain	Probably pathogenic	2	2/2	
15q26.2q26.3	chr15:96128092-100200967	4.1	Loss	Pathogenic ²	27	10/5	
15q26.3	chr15:98969215-102399819	3.4	Loss	Pathogenic ²	38	22/14	
16p11.2	chr16:29326560-30198151	0.9	Loss	Probably pathogenic	38	31/28	
16p13.3	chr16:247888-3061591	2.8	Gain	Probably pathogenic	146	117/86	
17p11.2	chr17:16578397-20234743	3.7	Loss	Pathogenic	107	49/38	Smith-Magenis syndrome
17q11.2q12	chr17:29533718-34346267	4.8	Loss	Pathogenic	80	56/46	NF1 microdeletion syndrome
17p13.3	chr17:148092-2363821	2.2	Loss	Pathogenic ²	50	38/33	Miller-Dieker syndrome
17p13.3	chr17:525-2294143	2.3	Loss	Pathogenic ²	50	38/34	Miller-Dieker syndrome
19q13.32	chr19:51842509-52739293	0.9	Gain	Probably pathogenic ²	43	30/16	
22q11.21	chr22:18626108-21798907	3.2	Loss	Pathogenic ²	96	49/44	22q11 deletion syndrome
22q11.21	chr22:18844632-21608479	2.8	Loss	Pathogenic	86	45/41	22q11 deletion syndrome
22q11.21	chr22:18886915-21463730	2.6	Loss	Pathogenic	82	44/40	22q11 deletion syndrome
22q11.21	chr22:19024793-21800471	2.8	Loss	Pathogenic	86	45/40	22q11 deletion syndrome
Xp22.33	chrX:61396-612228	0.6	Loss	Pathogenic	7	4/1	Leri-Weill dyschondrosteosis

MD, microdeletion; chr, chromosome; Mb, megabase; *n*, number; OMIM, Online Mendelian Inheritance in Man; UPD, uniparental disomy. ¹ Genomic positions are given according to Human Genome Building GCRh37, hg19. ² Confirmed as de novo CNVs.

Table 2. Characteristics of all the selected studies about CNVs in short stature patients of unknown cause as well as the present study

First author [Ref.]	Patients, <i>n</i>		aCGH/ SNPa	Patients' selection criteria
	total enrolled	with patho- genic CNV		
Zahnleiter et al., 2013 [8]	200	20 (10%)	SNPa	Short stature of unknown origin, either born with a normal birth size or born SGA, associated with dysmorphic features and/or developmental delay, but without criteria for the diagnosis of known syndromes
van Duyvenvoorde et al., 2014 [9]	142	17 (12%)	SNPa	Short stature of unknown origin, either born with a normal birth size or born SGA
Canton et al., 2014 [10]	51	8 (16%)	aCGH	Prenatal and postnatal growth retardation associated with dysmorphic features and/or developmental delay, but without criteria for the diagnosis of known syndromes
Wit et al., 2014 [11]	49	8 (16%)	SNPa	Short stature patients born small for gestational age
Present study	229	34 (15%)	aCGH/ SNPa	Short stature of unknown origin, either born with a normal birth size or born SGA, associated with dysmorphic features and/or developmental delay, but without criteria for the diagnosis of known syndromes

CNV, copy number variants; SNPa, single nucleotide polymorphism array; aCGH, array-based comparative genomic hybridization; SGA, small for gestational age.

Table 3. Recurrent submicroscopic genomic imbalances classified as pathogenic/probably pathogenic identified in 5 studies that investigated patients with short stature of unknown cause

Recurrent cases, <i>n</i> (%)	Locus	Common (critical) region	Size, Mb	Protein coding/ OMIM genes, <i>n</i>	Main candidate genes ¹	Ref.
9 (10.3)	22q11.21	chr22:21,011,217-21,440,656	4.3	9/9	<i>LZTR1, SNAP29</i>	8–11, present study
8 (9.2)	15q26	chr15:98,456,575-101,003,122	2.5	10/5	<i>IGF1R</i>	9, 11, present study
3 (3.4)	Xp22.33	chrX:61,396-612,228	0.5	4/4	<i>SHOX</i>	9, 11, present study
3 (3.4)	1p36.33	chr1:1,018,337-2,188,572	1.2	43/30	<i>B3GALT6, DVL1</i>	8, present study
3 (3.4)	UPD14	NA	NA	NA	NA	11, present study
2 (2.3)	1q21.1	chr1:146,101,228-147,831,171	1.7	10/10	<i>GJA5, GJA8, CHD1L, BCL9</i>	8
2 (2.3)	2q24.2	chr2:161,229,701-163,169,595	1.9	9/9	<i>IFIH1</i>	present study
2 (2.3)	17p13.3	chr17:148,092-2,294,143	2.1	37/33	<i>SERPINF1, DPH1, YWHAE, WDR81, VPS53</i>	present study

UPD, uniparental disomy; NA, not applicable; *n*, number; chr, chromosome; Mb, megabase; OMIM, Online Mendelian Inheritance in Man. ¹ Main candidate genes involved in growth impairment using the VarElect NGS Phenotyper Program.

In the present study, the prevalence of chromosome imbalances was 14.8%, showing their relevant contribution as a possible genetic mechanism in short stature. Other studies that investigated the impact of CNVs in children with short stature of unknown origin, regardless of physical or developmental defects, also identified a

high rate of pathogenic or probably pathogenic CNVs (10–16%) [8–11].

Recently, studies have recognized recurrent pathogenic or probably pathogenic CNVs associated with neurodevelopment disorders, ovarian cancer, and heart diseases, indicating that there might be some genetic “hotspots”

predisposing to different diseases [19–21]. Analyzing the 4 selected studies on short stature as well as the present study, 7 recurrent CNVs and 1 uniparental disomy were identified in patients with short stature of unknown cause. These CNVs were characterized as large, de novo, and individually rare, similar to those from other conditions in which there might be genetic “hotspots” for chromosomal imbalances [19, 20]. These loci corresponded to 40.2% of the total pathogenic or probably pathogenic CNVs described in all studies in which the short stature phenotype was the main emphasized phenotype.

In this study, we did not test inheritance in all patients. However, these patients had clear pathogenic, large CNVs (more than 3 Mb) and healthy parents. In 2010, a guideline about chromosomal microarray was published. In this consensus, the vast majority of inherited CNVs are described as much smaller than 500 kb, whereas most pathogenic CNVs are larger than 1 Mb and most occur de novo [7].

Among the recurrent CNVs identified in these studies, the 22q11 deletions were the most prevalent. The 22q11 deletion is described to be related to the DiGeorge syndrome (OMIM 188400)/velocardiofacial syndrome (OMIM 192430). This is one of the most common recurrent microdeletions in humans, with an estimated incidence of ~1:4,000 births. Although it is described as a well-known genetic syndrome, patients with 22q11 deletions usually have features with widely variable expressivity [22]. None of the patients with 22q11 deletion identified in studies of children with short stature of unknown cause have the typical signs associated with this syndrome [22, 23]. Also, several of these patients had some unspecific findings associated with 22q11 deletions (dysmorphic facial features, postnatal growth restriction, microcephaly, developmental and learning disabilities, psychiatric and/or behavioral problems) that could be confounded with other syndromes with a similar phenotype.

Another recurrent CNV identified was the 15q26 rearrangement. The insulin-like growth factor-1 receptor (*IGF1R*) gene, a well-known gene related to growth pathway, is located on chromosome 15q26.3. Haploinsufficiency of the *IGF1R* gene (OMIM 147370) is associated with impaired prenatal and postnatal growth [24]. Furthermore, there is a large spectrum of associated abnormalities, and it might be a confounding factor on clinical diagnosis [25–28]. The haploinsufficiency of the *IGF1R* gene has been described to be involved with developmental delay, facial and skeletal dysmorphisms, microcephaly, congenital heart disease, epilepsy, diaphragmatic her-

nia, renal anomalies, neonatal lymphedema, and aplasia cutis congenita [25, 26].

Xp22.33 rearrangement is also a common recurrent CNV, in which the *SHOX* gene is located, another well-known gene related to short stature [29, 30]. It has been mapped at the pseudoautosomal region 1 (*PAR1*) of sexual chromosomes X (Xp22.33) and Y (Yp11.2) [29, 30]. It is assumed that the heterozygous loss of the *SHOX* gene is responsible for Leri-Weill dyschondrosteosis (OMIM 127300), whereas homozygous loss leads to Langer mesomelic dysplasia (OMIM 249700), both being characterized by disproportionate short stature [29, 30]. Defects on the *SHOX* gene have also been identified in idiopathic short stature [31, 32]. Patients with identified Xp22.33 rearrangements usually had some additional features of disproportionate short stature that mask the suspicion of heterozygous loss of the *SHOX* gene.

Two recurrent CNVs were identified in chromosome 1: 1p36 (OMIM 607872) and 1q21.1 (OMIM 612474) deletions. They are among the most frequently described genomic disorders. The 1p36 deletion is characterized by craniofacial dysmorphism, developmental delay, and mental retardation [33], while in 1q21.1 deletion, the presence of psychiatric or behavior alterations and cardiac abnormalities are remarkable [34, 35]. Some clinical characteristics such as developmental delay, heart defects, seizures, skeletal anomalies, and short stature were described in both syndromes [33–37]. The wide phenotypic spectrum with variable penetrance and expressiveness and the overlapping of features make the clinical diagnosis more difficult.

Another recurrent variation found was a maternal uniparental disomy of chromosome 14. Although Temple syndrome (OMIM 616222) is a well-known short stature syndrome, the real incidence has probably been underestimated. The phenotype is variable, relatively mild, and age-dependent [38]. Both patients diagnosed with Temple syndrome in this study have a mild phenotype and a young age, which probably led to a misdiagnosis. These patients were clinically suspected of having Silver-Russell syndrome (SRS; OMIM 180860); however, both analyses for 11p15 ICR1 hypomethylation and uniparental disomy of chromosome 7 (UPD7) were negative. Some Temple syndrome features clinically overlap with SRS, including pre- and postnatal growth retardation, hypotonia, delay in the development of motor skills, and early puberty. Recently, the first SRS Consensus was published, and it suggested that Temple syndrome should only be investigated when SRS has been excluded due to the clinical overlap between both syndromes [39].

In this study, we used, SNPa and aCGH platforms, both proven to be efficient to detect copy number changes. However, it is known that SNP arrays have the advantage of also detecting stretches of homozygosity, which might represent uniparental disomy [7]. It is possible that if all studies had been done using SNP arrays, we would have more uniparental disomy diagnoses.

Two recurrent CNVs were exclusive for the present study: 17p13.3 and 2q24.2 rearrangements. The 17p13.3 rearrangement has been described in the Miller-Dieker lissencephaly (OMIM 247200). Although it is responsible for a well-defined syndrome, this diagnosis was not suspected for the patients. This syndrome is characterized by facial dysmorphisms, microcephaly, short stature, seizures, cardiac malformations, and, mainly, different severity grades of agyria in lissencephaly [40, 41]. However, our patients had some relatively nonspecific features and, most importantly, they do not have lissencephaly. The *LIS1* gene, responsible for lissencephaly and subcortical laminar heterotopia, was preserved in our patients. Regarding the 2q24.2 rearrangement, there are only few studies describing patients with mental retardation, generalized hypotonia, seizures, characteristic dysmorphic features, and delayed growth. In addition, other findings such as pulmonary emphysema have also been described [42]. Our 2 patients had some additional features, such as coloboma, scoliosis, microcephaly, and cardiopathy, showing a widely variable phenotype among these affected patients. In both cases, the atypical phenotype made the clinical diagnosis difficult.

In addition, there were 4 other studies about CNVs in short stature patients which did not fulfill our inclusion criteria, because either they used a custom genome-wide microarray or studied a specific growth disorder. Similarly, they also identified 6 of the 7 recurrent CNVs described in our study [43–46], indicating that these CNVs might be common in patients with the short stature phenotype.

In conclusion, chromosomal microarray made it possible to identify the etiology of syndromic short stature condition in 13% of the cases in whom the clinical approach was unable to establish a diagnosis due to relatively nonspecific features or a mild phenotype. Moreover, we observed some recurrent CNVs associated with this condition, corresponding to 40.2% of the total pathogenic or probably pathogenic CNVs described in all studies in which the short stature phenotype was the main emphasized phenotype. Among these recurrent CNVs, we identified deletions involving genes clearly associated with the short stature phenotype: *IGF1R* and *SHOX* [24, 29]. Additionally, novel candidate genes were suggested, and further studies should be performed to evaluate their role in growth disorders.

Statement of Ethics

Study was approved by the Ethics Committee for Analysis of Research Projects (CAPPesq) of the School of Medicine, University of Sao Paulo (USP) (#1645329).

Disclosure Statement

The authors declare that they have no competing interests.

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Supplemental table: Phenotype associated with short stature among the pathogenic or probably pathogenic CNVs.

Locus	Phenotype (in addition to short stature)
1p36.33	Microcephaly, developmental delay, motor and language disability, neonatal seizures, lordosis, respiratory allergies
1p36.33p36.32	Dysmorphic facial features, developmental delay, overweight
2p15p16.1	Dysmorphic facial features, developmental delay (motor, language), recurrent respiratory infections
2q24.1q24.2	Developmental delay, motor and language disability, seizures, scoliosis
2q24.2q24.3	Dysmorphic facial features, microcephaly, developmental delay, seizures, coloboma, recurrent pneumonias, hypothyroidism, cardiopathy, anterior ectopic anus
2q24.3q31.1	Dysmorphic facial features, developmental delay
2q37.2q37.3	Developmental disability, myopia, webbed neck
4p16.3p16.2	Dysmorphic global and facial features, microcephaly, global developmental delay, TDHA, recurrent infections, bone age delay
7q36.1q36.2	Dysmorphic facial features, global developmental delay, pectus excavatum, strabismus
8q23.3q24.13	Disproportional short stature (short extremities), sensorineural and conductive deafness, brachydactyly, thrombosis
9q22.2q22.33	Dysmorphic facial features, cleft palate and lip, microcephaly, developmental delay, socialization difficulty
10q22.2	Dysmorphic facial features, important bone age delay
10q26.3	Dysmorphic facial features, developmental disability
12q14.2q15	Dysmorphic facial features, microcephaly, developmental delay, strabismus
12q24.23q24.31	Dysmorphic facial features, developmental delay, hypothyroidism, hirsutism
14q24.3q32.33	Transitory hypotonia, oligodramnium and single umbilical artery on pregnancy
14q31.3q32.33	Dysmorphic facial features, transitory hypotonia, clinodactyly, pectus excavatum
15q11.1q11.2	Dysmorphic facial features, developmental delay
15q11.2q13.1	Dysmorphic facial features, cleft palate, developmental delay
15q13.3	Global developmental delay, language delay
15q26.2q26.3	Proportional short stature, microcephaly, important developmental disability
15q26.3	Microcephaly, developmental disability
16p11.2	Dysmorphic facial features, global developmental delay, gastroesophageal reflux, sacral dimple, syndactyly
16p13.3	Microcephaly, developmental delay, dilated Virchow-Robin spaces, ureteropelvic junction stenosis

17p11.2	Dysmorphic facial features, developmental delay, hypotonia, socialization difficulty, obesity
17q11.2q12	Dysmorphic facial features, macrocephaly, developmental delay (language and learning)
17p13.3	Dysmorphic facial features, short limbs, important developmental disability, hydrocephaly, thorax excavatum, bilateral cryptorchidism, sacral tuberosity, congenital heart disease (ventricular septal defect)
17p13.3	Dysmorphic facial features, proportional short stature, mild developmental delay, seizure, cryptorchidism clinodactyly, camptodactyly, trigger finger, lentiginose
19q13.32	Dysmorphic facial features, microcephaly, developmental delay
22q11.21	Disproportional short stature (short trunk), dysmorphic facial features, developmental delay, congenital heart disease (ventricular septal defect, pulmonary atresia)
22q11.21	Developmental delay, low weight
22q11.21	Dysmorphic facial features, sensorineural deafness, loss of strength, micropenis, stenosis of the auditory canal
22q11.21	Dysmorphic facial features, global developmental delay, agenesis of the corpus callosum
Xp22.33	Disproportional short stature (mezomelic)

CAPÍTULO 2 - *Rare genetic disorders in prenatal onset syndromic short stature identified by exome sequencing*

O diagnóstico etiológico das síndromes genéticas permanece um desafio. A raridade, heterogeneidade e variabilidade clínica torna o reconhecimento difícil mesmo dentre grupos especializados (7, 10). Estima-se que apenas metade ou menos dos pacientes que apresentam sinais dismórficos e/ou atraso do desenvolvimento tem o diagnóstico etiológico estabelecido clinicamente (43).

Atualmente, a avaliação de crianças com baixa estatura sindrômica é realizada mediante exames laboratoriais, de imagem, avaliação por especialistas e estudos genéticos para que o diagnóstico etiológico da baixa estatura seja estabelecido (17-19). O último *guideline* de avaliação de baixa estatura do *American College of Medical Genetics* (ACMG) preconiza que pacientes sindrômicos sejam avaliados por especialistas na tentativa de se identificar uma etiologia. Caso a etiologia seja detectada, pode-se fazer a confirmação molecular através de teste genético alvo. Nos casos em que esse diagnóstico clínico não é possível, preconiza-se a realização de cariótipo molecular (10).

Entretanto, como indicado por estudos que utilizaram essa metodologia na avaliação de crianças com baixa estatura (26, 40-42), o achado de variações no número de cópias explicaria apenas cerca de 10-16% dos casos. Diante disso, esse estudo buscou avaliar crianças com baixa estatura sindrômica de causa desconhecida nascidas pequenas para idade gestacional por sequenciamento exômico. Os dados desse estudo foram submetidos para publicação e estão aguardando avaliação.

Rare genetic disorders in prenatal onset syndromic short stature identified by exome sequencing

Short title: Genetic causes of syndromic short stature

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Abbreviations: SGA: small for gestational age; WES: whole exome sequencing; SDS: standard deviation score; MLPA: multiplex ligation-dependent probe amplification; SNVs: single nucleotide variants; IGV: Integrative Genome Viewer; LoF: Loss-of-Function; M: male; F: female; ACMG/AMP: The American College of Medical Genetics and Genomics and the Association for Molecular Pathology; ID: identity; Consang: consanguinity; SS: short stature; Y: years; NA: not available; IUGR: intrauterine growth retardation.

Abstract

OBJECTIVE: To perform a prospective genetic investigation using whole exome sequencing (WES) of a group of patients with syndromic short stature born SGA of unknown cause. **METHODS:** We selected 44 children born small for gestational age with persistent short stature, accompanied by additional features, such as dysmorphic facies, major malformation, developmental delay and/or intellectual disability, to be analyzed by WES. Seven patients had negative candidate gene testing based on clinical suspicion and thirty-seven patients had syndromic conditions of unknown etiology. **RESULTS:** Fifteen of 44 patients (34%) had pathogenic/likely pathogenic variants in genes already associated with growth disturbance: *COL2A1* (n=2), *SRCAP* (n=2), *AFF4*, *ACTG1*, *ANKRD11*, *BCL11*, *BRCA1*, *CDKN1C*, *GIN51*, *INPP5K*, *KIF11*, *KMT2*, and *POC1A* (one each). Most of the genes found to be deleterious participate in fundamental cellular processes, such as, cell replication and DNA repair. Many of these conditions have been described recently and/or have few patients described to date. **CONCLUSION:** The rarity and heterogeneity of syndromic short stature make the clinical diagnosis difficult. WES is effective in diagnosing a large number of previously undiagnosed patients with syndromic short stature.

Key words: growth disorder; short stature; small for gestational age; intrauterine growth retardation; syndrome; whole exome sequencing.

Introduction

Children with syndromic growth disorders born small for gestational age (SGA) represent a significant proportion of patients who are referred for pediatric or genetics evaluation. The correct diagnosis has important implications for genetic counseling, treatment and follow-up¹⁻⁴. The clinical diagnosis of these patients is a special challenge. There are almost four hundred genetic syndromes in which prenatal onset of growth retardation is one of the cardinal features^{5,6}. Each of these disorders presents distinctive clinical, laboratory and radiological characteristics, but due to their rarity, the diagnosis is difficult even by expert groups⁷⁻⁹. Additionally, several disorders present genetic heterogeneity, marked clinical variability or non-specific features, causing an overlap amongst different conditions, limiting the utility of a candidate gene approach to genetic diagnosis^{1,4,8,10}. The challenge has increased considerably with many recently described new genotype-phenotype correlations¹¹.

In this context, some authors have suggested the need to expand the genetic investigation for patients who lack a diagnosis based on standard approach that includes clinical evaluation, followed by additional phenotypic evaluation tailored to the patient's findings (for example: laboratory tests, karyotype, audiology, ophthalmology, cardiology and radiology evaluations)¹²⁻¹⁴. Studies using whole exome sequencing (WES) have shown a definitive diagnostic rate of 16-46% in patients with growth disorders of unknown etiology^{9,15-17}, however, none of them focused on syndromic children born SGA. Therefore, the purpose of this study was to investigate a group of short children with syndromic prenatal onset growth retardation of unknown etiology using WES. Our results demonstrated that WES is an effective tool to establish a definitive diagnosis in a significant proportion of children with syndromic growth disorders of prenatal onset.

Materials and Methods

Subjects

This study was approved by the local medical ethics committee. All patients and/or guardians gave written informed consent. The initial cohort comprised 187 patients born SGA [birth weight and/or length SDS ≤ 2 for gestational age] with persistent short stature [height standard deviation score (SDS) ≤ 2 at age of 2 years or above] and additional features, such as dysmorphic face, major malformation, developmental delay and/or intellectual disability (Figure 1). They were submitted to anthropometric analysis

and to a detailed history and physical examination by professionals with expertise in dysmorphology (D.R.B.; C.A.K; R.S.H; S.M.M.S. and/or A.A.L.J). Laboratory, radiographic assessment and genetic analysis were performed according to clinical indication.

Patients were classified as those with known ($n = 84$) or unknown short stature syndrome ($n=103$), according to clinical recognition of the condition (Figure 1). Among the recognized syndromic patients ($n=84$), we confirmed the diagnosis in 63 cases by genetic study (Supplemental table 1), 7 patients had a negative result in candidate gene approach, and 14 patients were not tested genetically either because they had specific criteria for clinical diagnosis ($n=7$) or due to unavailable DNA ($n=7$). Among the 103 patients with unknown short stature syndrome, 15 patients were excluded due to positive results by molecular karyotyping (Supplemental table 2), three for positive result in multigene panel sequencing (Supplemental table 3), and forty-eight due to unavailable DNA/consent term.

Therefore, we selected 44 children to be analyzed by WES following the inclusion criteria: available DNA samples; consent by the patient and family, and a negative result in candidate gene approach based on clinical suspicion ($n = 7$) or a syndromic condition without an initial clinical suspicion ($n = 37$). Among these 44 patients, thirty-seven had already been investigated by a genetic test prior to enrollment in the present study, mainly molecular karyotype ($n = 32$), multiplex ligation-dependent probe amplification (MLPA) for Silver-Russell syndrome investigation ($n= 3$) and targeted gene panel sequencing, which included genes frequently associated with growth disorders ($n = 7$).

Whole exome sequencing

Genomic DNA was extracted from peripheral blood leucocytes of all patients using standard procedures. In 23 patients, we analyzed the patient and both parents (trio analysis) and in 21, only the affected patient. We performed WES according to previously published protocols¹⁸. Briefly, the libraries were constructed with the SureSelect Target Enrichment System (Agilent Technologies, Santa Clara, USA) according to the manufacturer's instructions. The sequences were generated in the Illumina HiSEQ 2500 (Illumina, Inc, San Diego, CA, USA) platform running on paired-end mode. Reads were aligned with the GRCh37/hg19 assembly of the human genome with the Burrows-Wheeler Alignment (BWA-mem) aligner. Variant call included single nucleotide variants

(SNVs), small insertions and deletions (InDels) and it was performed by Freebayes. We used ANNOVAR to annotate the resulting data (in variant call format –VCF). The median coverage of the target bases was 102x with 97% of the target bases having ≥ 10 x coverage.

Data analysis

The exome data were screened for rare variants [MAF<0.1%] in public (gnomAD, <http://gnomad.broadinstitute.org/> and ABraOM <http://abraom.ib.usp.br/>) and in-house databases, located in exonic regions and consensus splice site sequences. Subsequently, our variant filtration prioritized genes based on their potential to be pathogenic: loss-of-function (LoF) variants (stop-gain; splice site disrupting; frameshift variants) and missense variants predicted to be pathogenic by multiple *in silico* programs. For variants identified by WES, we selected variants that fit based on different modes of inheritance (*de novo*, autosomal dominant, autosomal recessive and X-linked). The sequencing reads carrying candidate variants were inspected visually using the Integrative Genomics Viewer (IGV) to reduce false positive calls. The assessment of gene function was performed by OMIM® and the PubMed databases. Sanger sequencing and segregation were performed to validate the identified candidate variant. All variants were classified following the ACMG/AMP variant pathogenicity guidelines¹⁹.

Statistical analysis

Descriptive and comparative statistical analyses between the variables were conducted by using SigmaStat for Windows version 3.5 (SPSS Inc., San Jose, CA, USA). Differences between groups were tested by t-test or Kruskal–Wallis and Fisher exact test, as appropriate. Statistical significance was assumed for $P < 0.05$.

Results

The cohort was characterized by a male predominance ($n = 26$). Among these children with syndromic short stature, 36% were born SGA for both weight and length, 52% were SGA just for length and 6.8% were SGA just for weight (Table 1). At the first evaluation, they presented at a mean chronological age of 6.8 years and marked short stature (height SDS of -3.1 ± 1.3). The most recurrent phenotypes associated with short stature among these patients were dysmorphic facial features ($n = 36$; 82%), developmental delay and/or intellectual disability ($n = 26$; 59%), microcephaly ($n=21$; 48%), findings compatible with skeletal dysplasia ($n = 16$; 36%) and congenital heart disease ($n = 5$; 11%).

In total, we identified 15 (34%) pathogenic (n = 11) or likely pathogenic (n = 4) variants in genes already known as cause of syndromes associated with growth disturbance (Table 2). Most of these variants were heterozygous *de novo* variants (n = 9), followed by homozygous variants inherited in an autosomal recessive fashion (n = 2), heterozygous inherited from an affected parent (n = 2), and compound heterozygous variants (n = 1). We could not verify the inheritance pattern of one variant due to the absence of father's DNA. Eight variants were predicted to be protein-truncating (four frameshifts, three nonsenses and one variant located in a consensus splice site). All variants were absent in local and public databases (gnomAD and AbraOM); eleven of them were previously reported to be associated with short stature in the literature, and four variants were reported for the first time in this publication (*ACTG1*, *KIF11*, *KMT2A*, *POC1A*).

Patients harboring pathogenic variants or likely pathogenic variants had a heterogeneous phenotype (Figure 2; Table 3). Comparing the patients with positive and negative findings in WES, we did not find a correlation between the rate of diagnosis and the clinical characteristics analyzed (Tables 1 and 3). The definitive diagnosis based on the WES result can be classified into groups according to the molecular pathway involved¹³. Most of the disorders comprised a group of genetic defects in fundamental cellular processes, such as, cell replication and transcription (*ACTG1*, *AFF4*, *ANKRD11*, *BCL11B*, *GIN51*, *KIF11*, *KMT2A*, *POC1A*, *SRCAP*,) or DNA repair (*BRCA1*). Most patients with defects in these genes (10 of 11) had neurodevelopmental delay and/or intellectual disability as a hallmark. In contrast, two patients with genetic defects which affect cartilage extracellular matrix via a mutation in *COL2A1* were SGA only for length and had disproportionate short stature.

Discussion

The establishment of a precise diagnosis for children with syndromic short stature is very important for treatment decisions, correct prognostication, and for providing accurate genetic counseling for the affected patients and their families^{1, 3, 12}. Although there is no recent consensus about the investigation of syndromic short stature, these patients were usually submitted to a series of exams, evaluation by many specialists and genetic studies based on a candidate gene approach¹. A genomic approach, initially based on molecular karyotyping is recommended for children with unrecognized short stature

syndrome¹. Our group previously demonstrated that the molecular karyotyping analysis detected 15% of pathogenic/probably pathogenic CNVs in this context^{20, 21}.

In the present study, we investigated 44 syndromic children born SGA without a clinical diagnosis or negative results at initial genetic tests based on candidate gene approach by WES. We established the molecular genetic diagnosis in 15 of these patients (34%). This positive rate is similar to other studies that evaluated syndromic growth disorders (15.5 to 46.2%)^{9, 15-17}, however it is twice our recent findings in children born SGA with isolated short stature (15% vs 34%, $p = 0.031$)²². This result supports previous studies that suggest higher rates of positive diagnosis among patients with intellectual disability, major malformation, facial dysmorphisms, skeletal dysplasia, familial cases and/or parental consanguinity^{10, 12, 13, 17}. There were no significant differences in clinical features between groups with a positive or negative diagnosis, although our relatively small cohort limits the statistical power. Another study that analyzed 114 short stature patients by WES and molecular karyotyping found a higher diagnostic yield in patients with facial dysmorphism or skeletal abnormalities than in those without the corresponding phenotype¹⁷.

According to the classification of the genes by molecular mechanisms¹³, we can infer that variants found in genes belonging to fundamental cellular processes and intracellular pathways are most likely to present a syndromic phenotype. These genes can produce severe global growth deficiencies, which affect not just the growth plate but multiple other tissues and typically impair both pre- and postnatal growth, with neurodevelopmental delay/intellectual disability and another associated phenotype¹³.

We performed a retrospective analysis of all patients diagnosed by WES, and each one presented with clinical findings associated with the genetic diagnosis (Table 2). However, in most cases, diagnosis based on clinical features alone could be considered unlikely without a molecular genetic approach. Our patients reflect the rarity, heterogeneity and variability found in many syndromic conditions, that makes clinical recognition difficult. For example, one patient was diagnosed with SOFT syndrome (OMIM #614813), a condition with less than twenty cases reported to date²³. Another patient was diagnosed with a Fanconi Anemia-like syndrome (FA-like, OMIM #617883), being the third case published establishing the *BRCA1* variant as a cause of FA-like syndrome¹⁸. It shows how difficult the clinical diagnosis can be even in specialized groups due to the rarity of these conditions.

Other patients were not clinically recognized probably due to their young ages, such as the patients diagnosed with Baraitser-Winter syndrome 1 (OMIM #243310), and Wiedemann-Steiner syndrome (OMIM #605130). These syndromes are neurologic conditions that progressively evolve ²⁴⁻²⁶, and our patients were children younger than five years old. It is expected that it would be easier to make a clinical diagnosis as the children age, and the clinical signs become more evident.

Some patients had clinical findings that were not expected to be associated with the diagnosis. We identified two children with Floating Harbor syndrome (OMIM #136140) with additional characteristics not clearly associated with this condition that prevented prompt recognition, such as midline defects, epilepsy, high levels of IGF-1, and familial short stature. Another patient was diagnosed as KBG syndrome (OMIM #148050), however, this patient had a pancytopenia which is not clearly associated with this condition. Additionally, KBG is typically characterized as a postnatal short stature syndrome ^{27, 28}.

Some patients were clinically misdiagnosed, and as the target sequencing was normal, they were submitted to WES. One patient was initially suspected of Cornelia de Lange syndrome (CdLS) (OMIM #122470), and her WES identified a likely pathogenic variant in gene *AFF4* associated with CHOPS syndrome (OMIM #616368). Another patient was initially diagnosed as Silver Russel syndrome (OMIM # 180860), and her WES diagnosed a mutation in a paternally imprinted gene *CDKN1C* associated to IMAGE syndrome (OMIM #614732). These patients represent the phenotypic overlap among different syndromes.

The majority of the selected cases remain undiagnosed. Our positive rate among patients with clinically recognized short stature syndrome (58 of 65 patients investigated, 89%; Figure 1 and Supplemental Table 1) was significantly higher than patients with an unrecognized condition (15 of 44, 34%; $p < 0.001$), this difference can be partially explained by the presence of mutations in genes not yet associated with phenotypes. Additionally, these patients are likely to have epigenetic modifications, somatic mosaicisms and sequence variations in regions not targeted by WES ^{15, 29}. Nonetheless, our genetic approach was quite effective in establishing definitive diagnosis in a group of patients with unknown syndromic short stature. Despite the need for more evidence of the cost-effectiveness of WES ³⁰, we believe that obtaining genetic results can save time by ending the diagnostic odyssey of a wide range of exams and medical evaluations. Also,

it can provide accurate information for genetic counseling and alter medical management^{4, 31}. Therefore, our results support that patients with syndromic short stature born SGA of unknown cause should be evaluated by WES.

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Figure 1: Flowchart of the approach used in this study to investigate a group of patients with syndromic short stature.

Figure 2: Patients with syndromic short stature diagnosed by whole exome sequencing as: a) Baraitser-Winter syndrome 1; b) CHOPS syndrome; c) KBG syndrome; d) Intellectual developmental disorder with speech delay, dysmorphic facies, and t-cell abnormalities; e) Fanconi-Anemia like; f) Microcephaly with or without chorioretinopathy, lymphedema, or mental retardation; g) Wiedemann-Steiner syndrome; h) SOFT syndrome; i) Floating-Harbor; j) Floating-Harbor.

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Table 1 Clinical characteristics of patients with syndromic short stature analyzed by WES.

Characteristics	Total (N=44)	Positive diagnosis (N=15)	Negative diagnosis (N=29)
Sex (M/F)	26/18	9/6	17/12
Gestational age (weeks)	38 ± 3.0	38.2 ± 2.9	37.3 ± 3.5
Premature (%) ¹	7 (15.9)	-	7 (24.1)
Birth length SDS	-3.4 ± 1.3	-3.4 ± 1.4	-3.3 ± 1.4
Birth weight SDS	-1.9 ± 1.3	-1.9 ± 1.4	-1.9 ± 1.1
Birth head circumference SDS	-0.9 ± 1.8	-0.9 ± 1.8	-1.0 ± 1.9
Age (years)	6.8 ± 4.8	7.0 ± 4.9	6.5 ± 5.0
Height SDS	-3.1 ± 1.3	-3.2 ± 1.3	-3.1 ± 1.1
Target height SDS	-0.9 ± 1.1	-0.8 ± 1.0	-0.8 ± 1.1
Target height SDS – height SDS	-2.2 ± 1.7	-2.3 ± 1.8	-2.2 ± 1.6
Body mass index SDS	-0.7 ± 2.1	-0.9 ± 2.1	-0.9 ± 1.9
Abnormal body proportions (%) ²	18 (40.9)	7 (46.6)	11 (37.9)
Facial dysmorphism (%)	36 (81.8)	12 (80)	24 (82.7)
Intellectual disability/neurodevelopmental delay (%)	26 (59)	12 (80)	14 (48.3)
Microcephaly (%)	21 (47.7)	8 (53.3)	13 (44.8)
Major malformation (%)	17 (38.6)	7 (46.6)	10 (34.4)
Skeletal dysplasia (%)	16 (36.3)	6 (40)	10 (34.4)
Familial short stature (%) ³	15 (34.0)	4 (26.6)	11 (37.9)
Consanguinity (%)	10 (21.7)	4 (26.6)	6 (20.7)

WES: whole exome sequencing; M: male; F: female; SDS: standard deviation scores

1 – Birth that occurs before the 37th week of pregnancy.

2 – Assessed by sitting height/height ratio for age and sex.

3 – Familial history of short stature (family members with stature below -2 SDS for age and sex).

Table 2: Genetic diagnosis obtained by whole exome sequencing in 15 individuals with unrecognized syndromic short stature of prenatal onset.

ID	Gene	Genomic coordinate ¹	Variant ¹	Inheritance	Classification ²	Final diagnosis (OMIM)
1	<i>ACTG1</i>	Chr17:79478376	c.640G>A:p.Glu214Lys	<i>de novo</i>	Likely pathogenic	Baraitser-Winter syndrome 1 (243310)
2	<i>AFF4</i>	Chr5:132269985	c.772C>T:p.Arg258Trp	<i>de novo</i>	Likely pathogenic ³	CHOPS syndrome (616368)
3	<i>ANKRD11</i>	Chr16: 89350549 – 89350552	c.2398_2401del:p.Glu800Asnfs*62	<i>de novo</i>	Pathogenic ³	KBG syndrome (148050)
4	<i>BCL11B</i>	Chr14:99723991	c.242delG:p.Cys81Leu76fs	<i>de novo</i>	Pathogenic ⁴	Intellectual developmental disorder with speech delay, dysmorphic facies, and t-cell abnormalities (618092)
5	<i>BRCA1</i>	Chr17:41244839	c.2709T>A:p.Cys903*	Autosomal recessive	Pathogenic ⁵	Fanconi-Anemia like (604370)
6	<i>CDKN1C</i>	Chr11:2905900	c.820G>A:p.Asp274Asn	NA	Pathogenic ³	Intrauterine growth retardation, metaphyseal dysplasia, congenital adrenal hypoplasia, and genital anomalies (614732)
7	<i>COL2A1</i>	Chr12:48380136	c.1510G>A:p.Gly504Ser	<i>de novo</i>	Pathogenic ³	Spondyloepiphyseal dysplasia (120140)
8	<i>COL2A1</i>	Chr12:47984580	c.1852G>A:p.Gly618Ser	Inherited from affected mother	Pathogenic ³	Legg-Calve-Perthes disease (150600)
9	<i>GIN1</i>	Chr20:25397740 Chr20:25398748	c.139G>A:p.Val47Met c.247C>T:p.Arg83Cys	Autosomal recessive (Compound heterozygous)	Pathogenic ³	Immunodeficiency 55 (617827)
10	<i>INPP5K</i>	Chr17:1399973	c.1088T>C:p.Ili363Thr ⁸	Autosomal recessive	Likely pathogenic ³	Muscular dystrophy, congenital, with cataracts and intellectual disability (617404)

11	<i>KIF11</i>	Chr10:994405252 - 994405253	c.2400_2401del:p.His800fs	Inherited from affected father	Pathogenic	Microcephaly with or without chorioretinopathy, lymphedema, or mental retardation (152950)
12	<i>KMT2A</i>	Chr11:118376518	c.9911delT:p.Leu3304fs	<i>de novo</i>	Likely pathogenic	Wiedemann-Steiner syndrome (605130)
13	<i>POCIA</i>	Chr3:52183828	c.275+2T>G	<i>de novo</i>	Pathogenic	SOFT syndrome (614813)
14	<i>SRCAP</i>	Chr16:30737370	c.7330C>T:p.Arg2444*	<i>de novo</i>	Pathogenic ³	Floating-Harbor (136140)
15	<i>SRCAP</i>	Chr16:30737370	c.7330C>T:p.Arg2444*	<i>de novo</i>	Pathogenic ³	Floating-Harbor (136140)

1. GRCh37/hg19.

2. ACMG/AMP – The American College of Medical Genetics and Genomics and the Association for Molecular Pathology (18, 31).

3. Variants previously reported associated with short stature.

4. Variant previously described in a consortium of intellectual disability associated with *BCL11B* variants (32).

5. Variant previously described in a case report (17).

NA – Father's DNA unavailable.

Table 3: Clinical characteristics of the 15 patients with syndromic short stature diagnosed by whole exome sequencing.

ID	Genetic diagnosis (OMIM)	Sex	Consang	Familial SS	Birth length SDS	Birth weight SDS	Age (y)	Height SDS	Clinical phenotype
1	Baraitser-Winter syndrome 1 (243310)	M	-	-	-2.1	-0.5	3.0	-2.0	Dysmorphic face, strabismus, pectus excavatum, hypotonia, developmental delay, agenesis of corpus callosum and cerebellar vermis hypoplasia
2	CHOPS syndrome (616368)	F	+	-	-4.6	-3.9	17.5	-5.7	Microcephaly neurodevelopmental delay, facial dysmorphisms, congenital heart disease, recurrent pneumonias, obesity
3	KBG syndrome (148050)	M	-	-	-2.0	-1.7	16,4	-4,9	Microcephaly, seizures, hyperactivity, neurodevelopmental delay, polydactyly, umbilical hernia, macrodontia, strabismus, dysmorphic features, pancytopenia
4	Intellectual developmental disorder with speech delay, dysmorphic facies, and t-cell abnormalities (618092)	M	-	-	-2.0	-0.8	9.1	-2.1	Intellectual disability, speech impairment, delay in motor development, dysmorphic face, recurrent infections, asthma
5	Fanconi-Anemia like (604370)	F	+	-	-5.6	-4.4	2.5	-6.1	Microcephaly, neurodevelopmental delay, congenital heart disease and dysmorphic features
6	IUGR, metaphyseal dysplasia, congenital adrenal hypoplasia, and genital anomalies (614732)	F	-	-	-4.6	-3.8	8.2	-2.7	Initial diagnosis of atypical Silver-Russell syndrome, disproportional short stature, rhizomelia, cleft palate, umbilical hernia, clinodactyly
7	Spondyloepiphyseal dysplasia (120140)	F	-	-	-3.1	2.7	4.4	-3.6	Disproportionate short stature, important scoliosis
8	Legg-Calve-Perthes disease (150600)		-	+	-5.2	-1.2	11.8	-2.7	Disproportionate short stature and necrosis of capital femoral epiphysis
9	Immunodeficiency 55 (617827)	F	-	-	-4.5	-2.7	11.8	-3.2	Microcephaly, neurodevelopmental delay, recurrent respiratory and urinary infections,

10	Muscular dystrophy, congenital, with cataracts and intellectual disability (617404)	M	+	+	-3.8	-2.2	4.4	-3.9	uvula bifid, hypogonadism hypergonadotropic, ectopic kidney
11	Microcephaly with or without chorioretinopathy, lymphedema, or mental retardation (152950)	M	-	+	-3.2	-2.7	8.9	-2.0	Hypotonia, global developmental delay, microcephaly, cataract, and dysmorphic face
12	Wiedemann-Steiner syndrome (605130)	M	-	+	-4.1	-0.2	3.5	-2.6	Microcephaly, seizures, developmental delay, dysmorphic features
13	SOFT syndrome (614813)	M	+	-	-3.0	-2.8	8.7	-4.5	Microcephaly, intellectual disability, dysmorphic features, hairy elbows, high-arched palate, fifth finger clinodactyly
14	Floating-Harbor (136140)	M	-	+	-2.5	-1.4	5.3	-2.2	Disproportionate short stature; bone dysplasia, patent ductus arteriosus; low weight; hypermetropia and astigmatism; dysmorphic features
15	Floating-Harbor (136140)	F	-	+	NA	-2.6	9.0	-2.3	Microcephaly, neurodevelopmental delay, seizures, midline defect (cleft lip and palate), gastroesophageal reflux clinodactyly, cone-shaped phalangeal,

ID: identity; Consang: consanguinity; M: male; F: female; SS: short stature; SDS: standard deviation score; Y: years; NA: not available;
IUGR: intrauterine growth retardation

Supplemental table 1: Genetic diagnosis obtained by multiple genomic techniques in patients with recognized syndromic short stature.

Clinical diagnosis (OMIM#)	Gene / Region	Number of confirmed cases
Silver-Russell syndrome (OMIM #180860)	<i>11p15/ UPD(7)mat</i>	12
Noonan syndrome (OMIM #163950)	<i>PTPN11/SOS1/SHOC2</i>	7
Achondroplasia (OMIM #100800)	<i>FGFR3</i>	6
Hypocondroplasia (OMIM #146000)	<i>FGFR3</i>	4
Floating-Harbor syndrome (OMIM #136140)	<i>SRCAP</i>	4
Resistance to IGF1 (OMIM #270450)	<i>IGF1R</i>	4
Fanconi anemia (OMIM #227650)	<i>FANCA</i>	3
Bloom syndrome (OMIM #210900)	<i>BLM</i>	3
Leri-Weil dyschondrosthosis (OMIM #127300)	<i>SHOX</i>	3
Langer Mesomelic Dysplasia (OMIM #249700)	<i>SHOX</i>	2
Cornelia de Lange syndrome (OMIM #122470)	<i>NIPBL</i>	2
Laron syndrome (OMIM #262500)	<i>GHR</i>	1
3M syndrome (OMIM #273750)	<i>CUL7</i>	1
Osteogenesis imperfecta (OMIM #166200)	<i>COL1A1</i>	1
Spondyloepimetaphyseal dysplasia, strudwick type (OMIM #184250)	<i>COL2A1</i>	1
Pycnodysostosis (OMIM #265800)	<i>CTSK</i>	1
CHARGE syndrome (OMIM #214800)	<i>CHD7</i>	1
Neurofibromatosis type 1 (OMIM #162200)	<i>NF1</i>	1
Cartilage-hair hypoplasia (OMIM #250250)	<i>RMRP</i>	1
Seckel (OMIM #210600)	<i>PCNT</i>	1
Rubinstein-Taybi syndrome 1 (OMIM #180849)	<i>CREBBP</i>	1
Temple syndrome (OMIM #616222)	<i>UPD(14)mat</i>	1
DiGeorge syndrome (OMIM #188400)	<i>22q11</i>	1
Williams-Beuren syndrome (OMIM #194050)	<i>7q11.23</i>	1
Total	-	63

UPD: uniparental maternal disomy

Supplemental table 2: Description of the genomic imbalances classified as pathogenic/probably pathogenic obtained by molecular karyotyping in 15 patients with unrecognized syndromic short stature.

ID	Cytogenetic Position	Genomic position (hg 19) ¹	Size (Mb)	Gain/Loss	Pathogenicity	MD Syndrome ²	Reference ³
1	3q26.33-q27.2	chr3: 181910216-185599937	3.7	Loss	Pathogenic		Present study
2	4q28.2-q31.21	chr4:12943805-144588797	15.1	Loss	Pathogenic		(19)
3	8q24.22-q24.3	chr8: 133108784-146294098	13.2	Gain	Pathogenic		Present study
	15q26.2-q26.3	chr15: 97439378-102383473	4.9	Loss	Pathogenic		Present study
4	14q24.3q32.33	chr14:73972535-107287663	33.3	UPD	Pathogenic	Temple Syndrome	(20)
5	15q26.2q26.3	chr15:96128092-100200967	4.1	Loss	Pathogenic		(20)
6	15q26.3	chr15:98969215-102399819	3.4	Loss	Pathogenic		(20)
7	16q24.1-q24	chr16:84236094-88675901	4.4	Gain	Pathogenic		Present study
8	17p13.3	chr17:148092-2363821	2.2	Loss	Pathogenic	Miller-Dieker Syndrome	(20)
9	17p13.3	chr17:525-2294143	2.3	Loss	Pathogenic	Miller-Dieker Syndrome	(20)
10	22q11.21	chr22:18626108-21798907	3.2	Loss	Pathogenic	22q11 deletion syndrome	(20)
11	22q11.21	chr22:18894620-21440656	2.5	Loss	Pathogenic	22q11 deletion syndrome	(19)
12	3q27.1-q27.3	chr3: 183902785-187476327	3.5	Loss	Probably pathogenic		(19)
13	14q11.2-q12	chr14: 20889405-23647906	2.7	Gain	Probably pathogenic		(19)
14	20p13	chr20: 30892-429772	0.4	Loss	Probably pathogenic		(19)
15	Xq13.1-q13.2	chrX: 71553894-73461083	1.9	Gain	Probably pathogenic		(19)

1-Genomic positions are given according to Human Genome Building GCRh37, HG19.

2-Microdeletion (MD) syndromes

3-Genomic imbalances reported in previous studies.

chr: chromosome; Mb: megabase; UPD: uniparental disomy.

Supplemental table 3: Genetic diagnosis obtained by target panel sequencing in patients with unrecognized syndromic short stature.

Clinical diagnosis (OMIM#)	Gene	Number of confirmed cases
KBG syndrome (OMIM #148050)	<i>ANKRD11</i>	2
Kabuki syndrome 2 (OMIM #300867)	<i>KDM6A</i>	1

CAPÍTULO 3 - *Homozygous loss of function BRCA1 variant causing a Fanconi-anemia-like phenotype, a clinical report and review of previous patients*

Este artigo demonstra a importância e a complexidade do processo de investigação de uma criança com baixa estatura e características dismórficas. Nesse estudo, avaliamos uma criança que apresentava uma baixa estatura importante associada à microcefalia, atraso no neurodesenvolvimento, cardiopatia congênita, além de uma facies síndrômica.

Essa criança tinha sido avaliada por especialistas em dismorfologia clínica, já havia realizado vários exames laboratoriais e de imagem, e nenhuma síndrome genética tinha sido identificada. Devido baixa estatura importante, a criança foi encaminhada para avaliação e tratamento com hormônio de crescimento (rhGH) em nosso serviço.

A avaliação por sequenciamento exômico nesse caso foi essencial para o diagnóstico dessa paciente. O diagnóstico correto nos impediu de realizar uma iatrogenia instituindo o tratamento com rhGH para esta criança, além de permitir a realização de aconselhamento genético e um melhor seguimento para a criança e seus familiares.

Esse estudo foi publicado na *Eur J Med Genet.* 2018 Mar;61(3):130-133. doi: 10.1016/j.ejmg.2017.11.003 e foi incluído no banco de dados do OMIM®, ajudando a consolidar o gene *BRCA1* como uma das causas de Anemia de Fanconi (OMIM #617883 - FANCONI ANEMIA, COMPLEMENTATION GROUP S; FANCS).

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Homozygous loss of function *BRCA1* variant causing a Fanconi-anemia-like phenotype, a clinical report and review of previous patients

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ABSTRACT

Background: Fanconi Anemia (FA) is a rare and heterogeneous genetic syndrome. It is associated with short stature, bone marrow failure, high predisposition to cancer, microcephaly and congenital malformation. Many genes have been associated with FA. Previously, two adult patients with biallelic pathogenic variant in Breast Cancer 1 gene (*BRCA1*) had been identified in Fanconi Anemia-like condition.

Clinical report: The proband was a 2.5 year-old girl with severe short stature, microcephaly, neurodevelopmental delay, congenital heart disease and dysmorphic features. Her parents were third degree cousins. Routine screening tests for short stature was normal.

Methods: We conducted whole exome sequencing (WES) of the proband and used an analysis pipeline to identify rare nonsynonymous genetic variants that cause short stature.

Results: We identified a homozygous loss-of-function *BRCA1* mutation (c.2709T > A; p. Cys903*), which promotes the loss of critical domains of the protein. Cytogenetic study with DEB showed an increased chromosomal breakage. We screened heterozygous parents of the index case for cancer and we detected, in her mother, a metastatic adenocarcinoma in an axillary lymph node with probable primary site in the breast.

Conclusion: It is possible to consolidate the FA-like phenotype associated with biallelic loss-of-function *BRCA1*, characterized by microcephaly, short stature, developmental delay, dysmorphic face features and cancer predisposition. In our case, the WES allowed to establish the genetic cause of short stature in the context of a chromosome instability syndrome. An identification of *BRCA1* mutations in our patient allowed precise genetic counseling and also triggered cancer screening for the patient and her family members.

1. Introduction

Fanconi Anemia (FA) (OMIM #227650) is a rare and heterogeneous genetic syndrome, inherited in an autosomal recessive trait or X-linked (Fanconi anemia, complementation group B) (Wu, 2016). It is associated with short stature, bone marrow failure, high predisposition to cancer, microcephaly and congenital malformation (Petryk et al., 2015). In the cellular level, FA patients share underlying defects in their ability to process DNA lesions that interfere with DNA replication, what increases cellular sensibility to DNA interstrand crosslinks (ICLs)

agents, such as diepoxybutane (DEB) (Cantor and Brosh, 2014). There are 21 identified genes (*FANCA* to *FANCV*) associated with FA and FA-like phenotypes, encoding proteins that participate in the DNA repair (Wu, 2016; Schneider et al., 2015; Gueiderikh et al., 2017; Bogliolo and Surrallés, 2015). The pathway in which these genes are part of, is also known as the Fanconi Anemia/BRCA pathway (Solomon et al., 2015).

Recently, biallelic pathogenic *BRCA1* (OMIM #604370) variant has been identified in two adult patients with short stature, microcephaly, developmental delay and cancer predisposing. Both patients with Fanconi Anemia-like phenotype (FA-like) were compound

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heterozygous for pathogenic variants in *BRCA1* (Domchek et al., 2013; Sawyer et al., 2015). In the present study, we report a Brazilian female child, with a nonsense homozygous *BRCA1* variant identified during the investigation of syndromic short stature by whole exome sequencing (WES). These findings consolidate that biallelic loss-of-function *BRCA1* variants cause FA-like.

2. Patient and methods

2.1. Clinical report

The index case is a 2.5-year-old Brazilian female with a proportionate severe short stature [height standard deviation score (SDS) = -6.1], low body mass index (-4.6 SDS) and microcephaly (head circumference SDS = -7.9). The patient is the only child of health consanguineous (third cousins) Brazilian parents with normal height (mid parental height SDS 1.2). She had intrauterine growth retardation and was born full term with birth weight of 1630 g (-4.4 SDS), birth length of 39.5 cm (-5.6 SDS) and head circumference of 25 cm (-7.7 SDS). At birth, patient was diagnosed with atrial sept defect, which has been corrected surgically. She had failure to thrive and neurodevelopmental delay. Physical examination showed additional dysmorphisms: thickened earlobe; long eyelashes, full upper lip, big teeth, anteverted nostrils, bilaterally clinodactyly and hyperchromic spots on trunk and feet (Fig. 1a; 1b). Routine screening tests for short stature were normal, including normal blood count, IGF-1, karyotype, skeletal survey and abdominal ultrasound. She was evaluated by a geneticist without specific clinical diagnosis. For this reason, the patient was selected for WES analysis.

2.2. Molecular-genetic analysis

This study was approved by the local ethics committee and a parental written informed consent was obtained before initiating the genetics studies. DNA sample was extracted from peripheral-blood leukocytes using standard procedures. The proband underwent WES

according to previously published protocols (Sawyer et al., 2015; de Bruin et al., 2016). Briefly, the library was constructed with SureSelect Human All Exon V6 Kit (Agilent Technologies, Santa Clara, USA) according to the manufacturer's instructions. The exome library was sequenced on a HiSeq 2500 platform (Illumina, San Diego, USA) with the use of Hiseq SBS V4 cluster generation and sequencing kit (Illumina, San Diego, USA) running on paired-end mode. Reads were aligned to the hg19 assembly of the human genome with the bwa-mem aligner. Duplicated reads were flagged with the bammarkduplicates tool from biobambam2 (Tischler and Leonard, 2014). Variant calling was performed with Freebayes and the resulting VCFs were annotated with ANNOVAR (Wang et al., 2010).

Based on the family pedigrees indicating consanguinity (Fig. 1c), the exome data were screened for homozygous variants in the patient, in addition to having a minor allele frequency (MAF) of 0.1% in our in house sequencing data sets and in public databases (genomAD, <http://gnomad.broadinstitute.org/> and Abraom <http://abraom.ib.usp.br/>). Silent mutations were excluded and we inspected visually the candidate variants using the Integrative Genomics Viewer (IGV). The assessment of gene function was performed using the Online Mendelian Inheritance in Man (OMIM) and the PubMed databases. Sanger sequencing was performed to validate the candidate variant identified.

2.3. Chromosome breakage study with diepoxybutane (DEB)

Heparinized blood sample was obtained from patient for testing chromosomal instability. Peripheral blood lymphocytes were stimulated with phytohemagglutinin (Vitrocell) in two 72 h cultures, consisting in 2 mL de blood and 5 mL of culture medium (RPMI 1640, fetal bovine serum, penicilin and L-glutamin). In one of these cultures, after 24 h, the DNA cross-linking agent DEB (Aldrich Chemical Company, Inc) at the final concentration 0.1 $\mu\text{g/mL}$ was added. The other untreated culture was used as a control. Harvesting was done according to current protocols. Slides were stained with Wright's eosin methylene blue solution for analysis of chromosomal aberrations. For each culture, 40 metaphases were scored by the numbers of rearrangements, gaps

Fig. 1. (a, b) The patient displays facial dysmorphism, including microcephaly, thickened earlobe; long eyelashes, micropthalmia, upper lip full, big teeth, anteverted nose, bilaterally clinodactyly. (c) Pedigree of the family with *BRCA1*: c.2709T > A (p.Cys903*). Circles indicate females and squares indicate males. Slashes indicate death. An arrow indicates the proband. Shading indicates breast cancer. (d) Cytogenetic study with diepoxybutane showed chromosomal breakage.

and chromosomal breaks per cell (Auerbach, 1993). A score (breaks/cell) superior to 0.74 in DEB culture is considered positive for Fanconi anemia diagnosis in our laboratory.

3. Results

Average coverage of target exonic regions was 122x, with 98.5% of the targeted region covered more than 10x. We identified 17 rare homozygous variants located in exon or consensus splicing regions. Sixteen of them were considered as having low probability to be involved in the patient's phenotype due to the following reasons: variant predicted to be tolerated by the *in silico* tools; it is associated with a phenotype not observed in our patient and/or variant in genes that tolerate homozygous LoF mutations (Supplemental Table S1). The most significant finding was a nonsense variant at exon 10 of the *BRCA1* gene (NM_007294.3): c.2709T > A (p.Cys903*). This variant was predicted to result in loss of function (LoF) of the protein and was absent in local and public databases up to submission date (GenomAD and 1000 genomes). Sanger sequencing confirmed the variant as homozygous, inherited from heterozygous parents. According to the American College of Medical Genetics and Association for Molecular Pathology (ACMG-AMP) (Richards et al., 2015), this variant was classified as Pathogenic. After their molecular genetic results, the patient was submitted to cytogenetic study with DEB that showed an increased chromosomal breakage (Spontaneous breaks/cell = 0.12 and induced DEB breaks/cell = 1.90; Fig. 1d). We clinically screened parents of the index case for cancer and we detected an enlarged axillary lymph node in the mother. Subsequent radiologic exams showed a breast lesion classified as BIRADS 4. A biopsy confirmed a lymph node positive for metastatic adenocarcinoma due to probable subclinical breast cancer. The screening for the identified *BRCA1* mutation in other family members are ongoing.

4. Discussion

Fanconi Anemia is a genetic and clinical heterogeneous syndrome, characterized by developmental abnormalities, prenatal onset growth retardation, high predisposition to cancer, bone marrow failure and genomic instability (Schneider et al., 2015; Solomon et al., 2015; Zheng et al., 2013; Duxin and Walter, 2015). It is caused by rare biallelic mutation in one of 21 known FA genes (*FANCA* to *FANCV*) (Gueiderikh et al., 2017; Bogliolo and Surrallés, 2015; Palovcak et al., 2017). Patients with variants in *FANCO*, *RAD51* and *FANCS* genes, show absence of bone marrow failure and leukemia predisposition, and are named as Fanconi Anemia – like genes (Schneider et al., 2015; Bogliolo and Surrallés, 2015).

Although FA is a well-known genetic syndrome, this condition had not been suspected in our patient due to the absence of some cardinal signs such as radial ray abnormalities or evidence of bone marrow dysfunction. Only after the identification of homozygous pathogenic variant in *BRCA1* [c.2709T > A (p.Cys903*)] this diagnosis could be made. This is the first reported case with a homozygous *BRCA1* variant associated to FA-like phenotype. This nonsense mutation leads to a truncated protein, which lacks important functional regions, such as bind domain for interaction proteins, nuclear localization signal domain (NLS), a serine cluster domain (SCD) and the BRCA C- Terminal (BRCT) domain. Reviewing the literature, we found two other cases with FA – like caused by compound heterozygous for mutation in *BRCA1* (Domchek et al., 2013; Sawyer et al., 2015). All these three patients had microcephaly, short stature, developmental delay and dysmorphic face features (Table 1). However, differently from previous cases that were diagnosed only after breast or ovarian cancer, our patient was diagnosed in childhood during the investigation of short stature and before cancer development. *BRCA1* gene acts in the maintenance of genomic stability. Products of these genes comprise a network of DNA signaling and repair proteins, essential for maintenance of

genomic stability, known as Fanconi anemia/*BRCA1* pathway (Palovcak et al., 2017; Ceccaldi et al., 2016).

DNA inter strand crosslinks (ICLs) are DNA lesions that inhibit essential processes such as replication and transcription, and they must be repaired or bypassed for the cell to survive (Ceccaldi et al., 2016). ICL lesion repair during S and G2 phase of cell cycle requires FA pathway genes which orchestrate the steps of lesion recognition, DNA incision, lesion bypass and lesion repair (Ceccaldi et al., 2016). ICL recognition depends on the monoubiquitination of *FANCD2* and *FANCI*, in addition to a parallel function of homologous recombination (HR) proteins, such as *BRCA2* (also known as *FANCD1*) and *BRCA1* (also known as *FANCS*), responsible for recombination fork stabilization (Ceccaldi et al., 2016; Schlacher et al., 2012; Castella et al., 2015). It is well known that *BRCA1* is a cancer-predisposing gene. Mutations in *BRCA1* are associated to a familial risk of breast cancer (Stratton and Rahman, 2008) and heterozygous carriers of mutations in *BRCA1* have an 82% lifetime risk of breast cancer and a 54% of risk of ovarian cancer (King et al., 2003). In addition, it is associated to a higher risk of prostate, colon and pancreatic cancer and melanoma. For *BRCA1* mutation carriers, a close follow up for prevention and treatment decision-making is consistently recommended (Mohamad and Apffelstaedt, 2008; Moyer and Force, 2014).

Until now our patient has not developed cancer, however she is still very young and these complications might be present later. Assessment of maternal family history identified three women with breast cancer, including two sisters diagnosed before the age of 40 (Fig. 1c). Additionally, during follow up, we detected an enlarged axillary lymph node in the index's mother. She was submitted to screening for breast cancer that identified an undifferentiated metastatic carcinoma. Among the paternal family, we did not observe history of cancer, however almost all of them were males and it could be a protective factor to cancer, due to lower frequency of cancer by *BRCA1* variants in males.

In this case, the WES could help identify a genetic cause of short stature in the context of a defined chromosome instability syndrome. An identification of *BRCA1* mutations in our patient allowed precise genetic counseling and triggered cancer screening for the patient and her family members. The early detection and prompt diagnosis may avoid an invasive or most aggressive cancer. Moreover, the precise diagnosis of FA may prevent a wrong decision of treatment with recombinant human growth hormone (rhGH) for this patient. Unless a growth hormone deficiency (GHD) is convincingly documented, the treatment with rhGH for the short stature in these cases might be avoided. The long-term risk of rhGH treatment in FA patients is unknown. Nevertheless, no data suggest that cancer risk is further increased in FA patients treated with rhGH. The higher risk of malignancy itself in FA patients shows that one must be prudent to avoid the indiscriminate use of rhGH in these patients (Petryk et al., 2015).

In conclusion, our study demonstrates a novel homozygous nonsense *BRCA1* variant as a cause for Fanconi Anemia-like. Taken together all previous reports and the present one, it is possible to consolidate that biallelic loss of function *BRCA1* variant cause FA-like characterized by microcephaly, short stature, developmental delay, dysmorphic face features and cancer predisposition. In our case, the WES allowed to establish the genetic cause of short stature in the context of a chromosome instability syndrome. An identification of *BRCA1* mutations in our patient allowed precise genetic counseling and triggered cancer screening for the patient and her family members.

Disclosure summary

The authors declare there is no conflict of interest that could be perceived as influencing the impartiality of the research reported.

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Table 1Clinical features of the patients identified with pathogenic variants in *BRCA1* gene associated with Fanconi Anemia - like phenotype.

Publication	Domchek et al., 2013	Sawyer et al., 2015	Present study
BRCA1 genotype	Compound heterozygous variants	Compound heterozygous variants	Homozygous variant
cDNA level*	c.[2457delC]; [5207T > C]	c.[594,597del]; [5095C > T]	c.[2709T > A]; [2709T > A]
Protein level	(p.Asp821Ilefs*25); (p.Val1736Ala)	(p.Ser198Argfs*35); (p.Arg1699Trp)	(p.Cys903*)
Features at birth	No data available	Microsomia; dysmorphic features	Microsomia; dysmorphic features
Consanguinity	–	–	+
Gestational age	No data available	Full term	Full term
Birth length (cm) (SDS)	No data available	40.5 (–4.4)	39.5 (–5.6)
Birth weight (g) (SDS)	No data available	1990 (–3.5)	1630 (–4.4)
Birth head circumference (cm) (SDS)	No data available	27 (–5.6)	25 (–7.7)
Age (y)	28	25	2.5
Height (cm) (SDS)	150 (–2.1)	135 (–4.3)	68 (–6.1)
Body mass index (SDS)	No data available	21.9 (+0.1)	11.9 (–4.6)
Microcephaly	+	+	+
Neurodevelopmental delay	+	+	+
Radial ray abnormalities	–	+	–
Additional congenital abnormalities	No data available	Duodenal stenosis, hyper and hypopigmented skin lesions, slightly enlarged left kidney, conductive hearing loss 2–3 toe syndactyly, hyperextensible knees and history of hip dislocation	Atrial sept defect and hyperchromic spots on trunk and feet
Cancer	At age 28 with stage IV papillary serous ovarian carcinoma	At age 23 with ductal breast carcinoma	Not until now (age: 3.7years-old)
Bone marrow failure	No	No	No
Family history of cancer	Breast, ovarian and primary peritoneal cancer	Serous adenocarcinoma and ovarian cancer	Breast cancer

+ present, – absent; * reference sequencing NM_007294.3.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ejmg.2017.11.003>.

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Supplemental Table 1: 17 rare homozygous variant present at patient's exome.

Chr	Start	End	Ref.	Alt.	Gene	Effect on protein	Nucleotide:Aminoacid change	MAF – GenomAD	dbSNP ID	SIFT	Polyphen2	Mutation Assessor	PROVEAN	CADD
1	148009425	148009425	G	C	<i>NBPF14</i>	Nonsynonymous	c.1882C>G:p.Leu628Val	0	rs782087522	.	.	M	.	7,104
3	195511202	195511202	G	C	<i>MUC4</i>	Nonsynonymous	c.7249C>G:p.Leu2417Val	0	.	T	B	N	N	0.028
3	195511237	195511237	T	C	<i>MUC4</i>	Nonsynonymous	c.7214A>G:p.Asp2405Gly	0	.	T	P	N	N	0.221
3	195514811	195514811	C	T	<i>MUC4</i>	Nonsynonymous	c.3640G>A:p.Ala1214Thr	0	.	T	B	N	N	9,325
3	195514814	195514814	G	C	<i>MUC4</i>	Nonsynonymous	c.3637C>G:p.His1213Asp	0	.	T	B	N	N	0.181
3	195514819	195514819	G	T	<i>MUC4</i>	Nonsynonymous	c.3632C>A:p.Thr1211Lys	0	.	T	.	N	N	3,203
4	1102173	1102173	C	A	<i>RNF212</i>	Nonsynonymous	c.129G>T:p.Leu43Phe	0.0002	rs139291604	T	D	L	N	23.9
8	144946158	144946158	C	T	<i>EPPK1</i>	Nonsynonymous	c.1264G>A:p.Gly422Ser	0.0004	.	.	D	M	.	24.0
15	83011468	83011468	A	C	<i>GOLGA6L10</i>	Nonsynonymous	c.1600T>G:p.Cys534Gly	0	.	.	.	.	.	0
17	33814745	33814745	C	T	<i>SLFN12L</i>	Nonsynonymous	c.14G>A:p.Arg5Lys	0	.	T	.	N	N	14.36
17	34192286	34192286	C	A	<i>C17orf66</i>	Nonsynonymous	c.253G>T:p.Asp85Tyr	7.22E-06	.	.	P	N	.	11.47
17	34192369	34192369	T	C	<i>C17orf66</i>	Nonsynonymous	c.170A>G:p.Glu57Gly	0.0002	rs148728671	.	D	N	.	21.9
17	39464991	39464991	A	T	<i>KRTAP16-1</i>	Nonsynonymous	c.515A>T:p.Val172Glu	0.0002	.	D	.	L	N	16.83
17	41244839	41244839	A	T	BRCA1	Stop gain	c.2709T>A:p.Cys903*	0	.	.	.	.	.	35
17	42981735	42981735	C	T	<i>FAM187A</i>	Nonsynonymous	c.538C>T:p.Arg180Cys	7.14E-06	.	D	D	.	D	34
17	44110775	44110775	A	C	<i>KANSL1</i>	Nonsynonymous	c.271T>8G:p.Asn906Lys	0.0003	rs139615350	T	B	L	N	9,953
19	46443476	46443476	C	G	<i>NOVA2</i>	Nonsynonymous	c.1124G>C:p.Gly375Ala	0	rs545814576	T	B	N	N	0.001

Chr: chromosome; Ref: reference; MAF: minor allele frequency; GenomAD: Genome Aggregation Database; SIFT: Sorting Intolerant From Tolerant; Polyphen2: Polymorphism Phenotyping v2; PROVEAN: Protein Variation Effect Analyzer; CADD: Combined Annotation Dependent Depletion.

CAPÍTULO 4 - *Multigene sequencing analysis of children born small for gestational age with isolated short stature*

O termo pequeno para idade gestacional (PIG) descreve aqueles neonatos que apresentam escore-Z de peso e/ou comprimento ao nascer ≤ -2 para idade gestacional, de acordo com valores de referência populacionais (44, 45). Três a 10% dos recém-nascidos são PIG (45) e vários fatores já foram relacionados à essa condição, como causas fetais, placentárias, maternas e ambientais (44-47).

Na maioria dos casos, esses pacientes apresentam recuperação da estatura após o nascimento. Entretanto, cerca de 10 a 15% desses neonatos não apresentam *catch-up* espontâneo até os dois/quatro anos de idade (44-47). A persistência da baixa estatura num contexto de exclusão de causas gestacionais e excluídas outros interferentes ambientais podem sugerir uma causa genética (10, 45-48).

Considerando a heterogeneidade dos pacientes nascidos PIG, deve-se julgar que o termo PIG é, essencialmente, uma descrição de um aspecto fenotípico, e não um diagnóstico. E o estabelecimento preciso desse diagnóstico é importante para o prognóstico, seguimento e aconselhamento genético (8-10).

Portanto, a proposta do estudo foi investigar um grupo de pacientes com baixa estatura isolada, nascidos pequenos para idade gestacional através de painel gênico e/ou sequenciamento exômico a fim de identificar possíveis causas genéticas responsáveis pelo fenótipo.

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Multigene sequencing analysis of children born small for gestational age with isolated short stature

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Genetic investigation of nonsyndromic SGA children

Multigene sequencing analysis of children born small for gestational age with isolated short stature

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Context: Patients born small for gestational age (SGA) who present with persistent short stature may have an underlying genetic etiology which accounts for prenatal and postnatal growth impairment. We have applied a unique massive parallel sequencing approach in cohort of exclusively nonsyndromic SGA patients to simultaneously interrogate for clinically significant genetic variants.

Objective: To perform a genetic investigation of children with isolated short stature born SGA.

Design: Screening by exome (n=16) or targeted gene panel (n=39) sequencing.

Setting: Tertiary referral center for growth disorders.

Patients and Methods: We selected 55 patients born SGA with persistent short stature without an identified cause of short stature.

Main outcome measures: Frequency of pathogenic findings.

Results: We identified heterozygous pathogenic or likely pathogenic genetic variants in eight of 55 patients, all of them in genes already associated with growth disorders. Four of the genes are associated with growth plate development, *IHH* (n=2), *NPR2* (n=2), *SHOX* (n=1), and *ACAN* (n=1), and two of the genes are involved in the RAS/MAPK pathway, *PTPN11* (n=1) and *NFI* (n=1). None of these patients had clinical findings that allowed a clinical diagnosis. Seven patients were SGA only for length, and one patient was SGA for both length and weight.

Conclusion: These genomic approaches identified pathogenic or likely pathogenic genetic variants in eight of 55 patients (15%). Six of the eight patients carried variants in genes

associated with growth plate development, indicating that mild forms of skeletal dysplasia may be a cause of growth disorders in this group of patients.

In this study, the molecular investigation, using massively parallel sequencing, showed effectiveness in diagnosing one in seven patients with prenatal short stature of unknown etiology.

Introduction

Children born small for gestational age (SGA) for length and/or weight with persistent short stature during childhood have a high probability of having height below the normal range in adulthood (1). Usually, after an extensive medical evaluation, the cause of this growth disorder is not identified, and therapy with recombinant human growth hormone (rhGH) may be indicated to promote improvement in adult height (1-3).

The regulation of prenatal growth and the lack of catch-up growth in SGA children are not fully understood. The causes of SGA are multifactorial, including maternal/uterine-placental factors, fetal epigenetics and genetic abnormalities (2). Numerous genes have been associated with the regulation of human height and implicated in growth disorders (4,5). Several case reports (6,7) and candidate gene studies (8-10) have demonstrated that a percentage of children born SGA have a monogenic condition that explains their short stature (2).

Knowledge of the genetic basis for the growth disorders in these children has important consequences for their treatment and follow-up. For example, a broad genetic test allows an early recognition of mild forms of genetic conditions that increase the risk for cancer, such as Bloom syndrome, where rhGH therapy is contraindicated (7). Children with *IGF1R* haploinsufficiency have a variable phenotype that hinders their diagnosis based only on clinical characteristics (11,12). Nevertheless, the recognition of this genetic defect explains an elevated IGF-1 level associated with a poor response to rhGH therapy (13). Finally, identification of a heterozygous defect in *ACAN* allows the prediction of accelerated bone maturation and short growth spurt (9). Additionally, all of these positive genetic tests have significant implications in genetic counseling and early recognition of additional cases in the affected families (14).

The advent of new genomic technologies, especially massively parallel sequencing, has provided a genetic diagnosis in many children with short stature of unknown cause, especially among patients with syndromic conditions (15-18). However, no study has thus far focused on children born SGA with isolated short stature. Consequently, this current study aimed to evaluate the diagnostic yield of massive parallel sequencing approaches of the whole exome sequencing (WES) and/or targeted panel sequencing in a cohort of 55 nonsyndromic children born SGA without catch-up growth.

Patients and methods

Patients

We enrolled children born small for gestational age [birth length or weight SD score (SDS) ≤ -2] (19) referred for evaluation of short stature. Patients underwent a clinical assessment, and the inclusion criterion was persistent short stature (height SDS ≤ -2) after the second year of life without any known cause. We excluded patients with characteristics in addition to short stature, such as several dysmorphic features, major malformations, microcephaly, neurodevelopmental delay, intellectual disabilities or skeletal dysplasia. We did not exclude patients with short stature and minor malformations, defined as an unusual morphologic feature found in the general population causing no serious medical or cosmetic significance to the affected individual (20). Additionally, children born late preterm were also included (gestational age above 34 and below 37 weeks). Fifty-five patients were selected from an

initial cohort of 297 children born SGA without catch-up growth (Figure 1). Two of the patients were previously reported in studies that did not involve the evaluation of children born SGA (21,22).

The clinical assessment was performed at the same period of the day and included measurements of weight (measured on an electronic scale), standing height and sitting height (mean of three measurements on a calibrated stadiometer to the nearest 0.1cm). Body mass index (BMI) was calculated (weight/height²). These results were converted to SDS using age- and gender-specific norms (23,24). Height of the parents were obtained during the patients' visit by the same method in 85% of the cases. The anthropometric data from fourteen fathers was obtained by medical records reported by the family. We do not have access to the height of three fathers. Target height was calculated [(father's height + mother's height ± 13 cm)/2] and expressed as SDS. Left hand and wrist x-rays for bone age (BA) determination was assessed by the method of Greulich and Pyle (25).

All procedures were in accordance with the ethical standards of the Hospital das Clinicas Ethics Committee (CAPPesq) and the Helsinki Declaration. Consent was obtained from each patient and parent after full explanation of the purpose and nature of all procedures used.

Massive parallel sequencing

Targeted panel and exome sequencing

Genomic DNA was isolated from peripheral blood leucocytes from all patients, 36 mothers and 25 fathers using standard procedures. Target panel sequencing (n = 39) or WES (n = 16) was performed according to availability. Target panel sequencing was performed only for index patients, and WES was also performed for five of these patients. Among the other eleven patients submitted to WES, four had trio analysis. An autosomal dominant short stature was suspected in the other seven patients; therefore, in these families, WES was performed in the index case, the affected parent and additional relatives.

We developed customized target panel sequencing (Agilent SureSelect XT assay; Agilent Technologies, Santa Clara, CA, USA) that included 388 genes (Supplementary Table 1) (26). The selected genes were genes associated with short stature (growth-related genes derived from OMIM® and MedGen databases), genes involved in *GH-IGF1* pathway regulation, and candidate genes to be associated with growth disorders based on our previous results (27) and unpublished data. An exome library was prepared according to Sure Select Human All Exon V5 or V6 (Agilent Technologies, Santa Clara, CA, USA).

Sequencing was performed on an Illumina NextSeq 500 (Illumina, San Diego, CA, USA) platform for the targeted gene panel and a HiSeq 2500 (Illumina, San Diego, CA, USA) platform for the exome, both in paired-end mode. In-house bioinformatic analysis was performed as previously reported (28). The sequences were aligned with the human reference assembly (GRCh37/hg19).

Variant assessment

The exome and the targeted panel sequencing data were screened for rare variants (MAF<0.1%) in public international and national (gnomAD, <http://gnomad.broadinstitute.org/>, and ABraOM <http://abraom.ib.usp.br/>) and in-house databases, located in exonic regions and consensus splice site sequences. Subsequently, our variant filtration prioritized genes based on their potential to be pathogenic, including loss-of-function (LoF) variants and variants predicted to be pathogenic by multiple *in silico* programs (n = 7, Supplementary Table 2) (29). For variants identified by WES, we selected those based on different modes of inheritance (autosomal dominant *de novo*, autosomal recessive, and X-linked) according to the possible model of inheritance of the family. The sequencing reads carrying candidate variants were inspected visually using the Integrative Genomics Viewer (IGV) to reduce false positive calls. The assessment of gene function was performed by

OMIM[®] and the PubMed databases. Sanger sequencing and segregation were performed to validate the candidate variant identified.

All variants were classified following the American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) variant pathogenicity guidelines (30).

Results

Description of the cohort

We analyzed 55 patients born SGA without catch-up growth. The clinical characteristics of the studied group are shown in Table 1. The age of the first evaluation ranged from 2 to 16.3 years of age. The cohort was characterized by a male predominance (64%) and by a family history of short stature (69%). In seven patients, we did not have access to their length at birth (13%); among the other 48 patients, 25% were SGA for both weight and length, 55% were SGA just for length, and 7% were SGA only for weight. Nine patients (16%) had disproportionate short stature defined by sitting height/height ratio above 2 SDS from the mean (31). All these patients had a normal skeletal survey with only nonspecific findings. Among these disproportionate short stature children, six were SGA just for length, and three were SGA for length and weight.

Variants identified by massive parallel sequencing

We identified eight heterozygous pathogenic (n = 3) or likely pathogenic (n = 5) variants in eight of 55 patients (15%). All these variants were identified in genes already associated with growth disorders (Table 2): *Indian hedgehog* (*IHH* = 2); *Natriuretic peptide receptor 2* (*NPR2* = 2); *Short stature homeobox* (*SHOX* = 1); *Aggrecan* (*ACAN* = 1); *Neurofibromin 1* (*NF1* = 1) and *Protein-tyrosine phosphatase nonreceptor-type 11* (*PTPN11* = 1). Four variants were found by targeted panel sequencing and four by WES. All variants were absent or extremely rare in local and public databases. Five variants were inherited from affected parents. One of the variants was in a consensus splice site, and all the others were missense and predicted to be pathogenic by multiple *in silico* tools.

Phenotype of patients with pathogenic or likely pathogenic variants associated with short stature

All eight patients with pathogenic variants were born at full term. Seven patients were SGA only for length, and one patient was SGA for both length and weight. Six of the patients with pathogenic variants had a family history of short stature, four of them inherited the pathogenic variant from an affected parent. Two patients had minor malformations (Table 3). Six of eight patients had pathogenic variants in genes directly involved in growth plate development. Among these patients, two had body disproportion (*IHH* and *ACAN*). The skeletal survey depicted shortening of the middle phalanx of the 2nd and 5th fingers with cone-shaped epiphyses in both patients with *IHH* variants. Additionally, two patients had a slightly advanced bone age at prepubertal age (bone age SDS of 0.6 and 0.7 for a patient with *NPR2* and *ACAN*, respectively), whereas 3 patients had remarkably delayed bone age (bone age SDS of -4.4, -4.8 and -5.8 for a patient with *SHOX*, *PTPN11* and *NF1*, respectively) (Table 3).

Two patients had pathogenic variants in genes involved in the RAS-MAPK pathway (*PTPN11* and *NF1*). At the first evaluation, none of them had characteristics that allowed the diagnosis of Noonan syndrome (OMIM #163950) or neurofibromatosis type 1 (OMIM #162200). The patient with the *NF1* mutation had two small *café au lait* spots and normal ophthalmologic evaluation at 7 years old when he started follow-up in our clinic. Ten years later, at 17 years of age, he developed a plexiform neurofibroma, confirming the clinical diagnosis.

Discussion

Evaluation of children born SGA with persistent short stature and absence of syndromic features is a common challenge for both pediatricians and pediatric endocrinologists. Usually, the clinical evaluation, followed by traditional investigation using radiological and laboratory exams, is unable to determine a cause for pre- and postnatal growth impairment. The frequent observation of short stature in other first-degree family members suggests a genetic component. However, the absence of specific clinical findings makes impractical the use of a candidate gene approach. Consequently, our multigene strategy using next-generation sequencing was able to diagnose 15% of patients born SGA with persistent short stature without other characteristics.

There is a limited number of studies that applied the genomic approach to investigate short stature children. Usually, these studies comprised patients with short stature of unknown cause, regardless of birth weight or length, also including syndromic conditions (16-18,32). Pathogenic or likely pathogenic variants were observed in 2% to 46% of these children (16-18,32,33). This wide range of variability could be partially explained by the sequencing methods applied, the number of patients analyzed, and the inclusion/exclusion selection criteria of patients. A recent well-conducted study that evaluated 200 patients with growth disorders (134 classified as isolated short stature) by WES identified pathogenic variants in known short-stature genes in 21% of syndromic cases and 14.2% of isolated short stature, with a marked preponderance of autosomal dominant inheritance (18). This result was similar to that observed in our study, which analyzed only nonsyndromic children born SGA.

Among the patients diagnosed in the present study, all were SGA for length, and genes that participated in growth plate development were overrepresented (*SHOX*, *ACAN*, *NPR2*, and *IHH*). Our patients represent a mild variation of a skeletal dysplasia phenotype. Curiously, four patients inherited the pathogenic variant from an affected, but previously undiagnosed, parent. The role of these growth plate-associated genes in explaining the persistence of short stature in children born SGA has not been fully investigated. Mutations in these genes have already been associated with distinct degrees of short stature with or without other mild features, such as slight disproportional short stature and nonspecific skeletal abnormality (21,31,34-36). In many studies, birth weight and length were not reported. Each of these genes explains a small fraction (1-4%) of patients classified as idiopathic short stature (ISS) (21,36-40), with a higher prevalence in familial cases (40). Only one study reported the specific prevalence of *ACAN* gene mutations in children born SGA. From a cohort of 290 SGA children, 29 with advanced bone age were selected for gene sequence, and four patients (13.8%) were identified with *ACAN* mutations. Three of these patients were born SGA for length (9).

In our study, we also identified two patients with pathogenic variants affecting the RAS-MAPK pathway. One of our patients was diagnosed with neurofibromatosis type 1 (NF1) (OMIM #162200). Usually, the diagnosis of NF1 is made in children older than eight years old based on clinical features (41), but it may be difficult to diagnose young children. Our patient had an atypical presentation that delayed the clinical diagnosis until he was 17 years of age, when he developed a plexiform neurofibroma. In another patient, we identified a pathogenic variant in *PTPN11* associated with Noonan syndrome (OMIM #163950). Usually, Noonan syndrome is diagnosed by specific clinical criteria (42). The facial features and/or typical cardiac malformation frequently trigger the suspicion of the diagnosis. However, the patient analyzed in this study had a mild phenotype and did not meet the criteria for a clinical diagnosis. A recent functional study of the same variant found in this patient (*PTPN11* p.Arg265Gln) demonstrated an increased catalytic activity of the phosphatase and hyperactivation of the MAPK pathway signaling when stimulated by a growth factor

(Epidermal Growth Factor-EGF). It was responsible for a mild phenotype without the typical characteristics that usually compound the clinical picture of Noonan syndrome (43). Other studies using the genomic approach in patients with isolated short stature also identified *NFI* and *PTPN11* mutations in patients without typical clinical features (18,32).

The early diagnosis provided by a genomic approach has a significant impact on familial genetic counseling, rhGH treatment decisions, and patient follow-up. In our study, all affected patients have an autosomal dominant form of growth disorders with a recurrence risk of 50% for the patients' offspring and for the siblings of affected parents. The molecular diagnosis has therapeutic implications since hGH treatment seems effective in improving height of patients with *SHOX*, *ACAN* and *IHH* gene defects (9,21,44). Additionally, children with Neurofibromatosis type 1 should have a multidisciplinary approach to optimize their follow-up and medical care, early recognition of complications and to promote general health (41).

Although we do not find differences between rates of diagnosis using WES or target panel sequencing, the WES strategy permits the identification of new genes and allows a hypothesis-free method to assess for genetic variation (46). Even so, all genes identified with pathogenic variants by WES were present in our target gene panel. Nevertheless, recent studies that prospectively compared the cost-effectiveness between clinical WES and the traditional investigation showed a higher rate of diagnosis and lower cost when WES was used (15). A recent systematic review offers encouraging evidence of the cost-effectiveness of genomic sequencing over usual care for pediatric patients (47). The use of a genomic approach significantly increased the diagnostic rate to 16–79% and lowered the cost by 11–64% compared to the standard diagnostic pathway (47).

In this study, molecular investigation, using massively parallel sequencing, was effective in diagnosing one in seven patients with prenatal short stature of unknown etiology. All of these patients were clinically investigated extensively, and all were classified as children born SGA with isolated short stature. Without this genomic approach, these patients would have remained undiagnosed and probably have had an inadequate follow-up. As a karyotype is indicated to investigate all girls with unexplained growth failure (48), we believe that with a higher availability and lower cost of the genetic analyses, multigene molecular sequencing may be incorporated for investigation of all children born SGA with persistent short stature. Our results should encourage prospective studies that include patient well-being and economic cost-benefit evaluations to guide future recommendations for genetic testing using a genomic approach for children born SGA.

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Disclosure summary:

The authors declare there is no conflict of interest that could be perceived as influencing the impartiality of the research reported.

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Figure 1: Strategy used to select children born small for gestational age (SGA) with isolated short stature of unknown cause included in this study. IUGR = Intrauterine growth restriction.

Table 1: Characteristics of the 55 patients born SGA with isolated short stature involved in this study.

Characteristic	SGA patients
Consanguinity (%)	3 (5.5%)
Familial short stature ¹ (%)	38 (69.1%)

Sex (M:F)	35:20
Gestational Age (weeks)	39.1 ± 1.1
Premature ² (%)	6 (10.9%)
Birth weight SDS	-1.7 ± 0.7
Birth length SDS	-3.0 ± 0.2
Age at the 1 st evaluation (years)	9.7 ± 3.5
Height SDS	-2.7 ± 0.5
Sitting height : total height ratio SDS	0.8 ± 1.0
BMI SDS	-0.7 ± 1.0

M: male; F: female; BMI: body mass index.

1 – First degree family members with height SDS < -2

2 – Birth before the 37th week of pregnancy.

Continuous variables are shown as mean ± SD and numeric variables are shown as absolute number (percentage).

Table 2: Pathogenic and likely pathogenic variants identified by whole exome and targeted panel sequencing in a cohort of 55 patients born SGA with isolated short stature.

ID	Gene	Variant	Frequency (MAF) ³	Functional annotation	Inheritance pattern	ACMG/AMP
1	<i>IHH</i>	c.446G>A:p.Arg149His ¹	0	Missense	Inherited from affected mother	Likely Pathogenic ⁶
2	<i>IHH</i>	c.172G>A:p.Glu58Lys ¹	0	Missense	Inherited from affected father	Likely Pathogenic ⁶
3	<i>NPR2</i>	c.1249C>G:p.Gln417Glu ²	0.000004	Missense	Unavailable ⁴	Pathogenic ⁶
4	<i>NPR2</i>	c.94C>A:p.Pro32Thr ²	0	Missense	Inherited from affected mother	Likely Pathogenic
5	<i>PTPN11</i>	c.794G>A:p.Arg265Gln ²	0.00003	Missense	Unavailable ⁴	Pathogenic ⁶
6	<i>SHOX</i>	c.503G>A:p.Arg168Gln ²	0	Missense	Inherited from affected mother ⁵	Likely Pathogenic
7	<i>ACAN</i>	c.532A>T:p.Asn178Tyr ¹	0	Missense	Inherited from affected mother	Likely Pathogenic ⁶
8	<i>NFI</i>	c.1261-1G>C ¹	0	Splice site acceptor	Unavailable ²	Pathogenic

1. Result obtained from exome sequencing; 2. Result obtained from targeted panel sequencing; 3. MAF (minor allele frequency) based on gnomAD; 4. DNA from one of the parents was not available; 5 Heterozygous from mosaic mother; 6 Variants previously reported associated with short stature

Table 3: Clinical characteristics at the first evaluation of the patients with pathogenic or likely pathogenic variants identified by exome or targeted panel sequencing.

ID	Gene	Sex	Birth weight g (SDS)	Birth length cm (SDS)	Age (years)	Bone age (years)	Height SDS	SH:H SDS	BMI SDS	Mother height SDS	Father height SDS	Additional features
1	<i>IHH</i>	F	2785 (-0.9)	44 (-3.2)	8.0	6.8	-2.0	+3.4	-0.8	-2.6*	2.3	
2	<i>IHH</i>	M	2965 (-1.0)	46 (-2.5)	5.6	NA	-3.0	+0.6	+0.3	-1.6	-4.3*	
3	<i>NPR2</i>	F	2600 (-1.8)	46 (-2.5)	10.6	11.1	-2.0	+1.3	+1.1	-1.5	-0.7	
4	<i>NPR2</i>	F	3310 (-0.2)	46 (-2.5)	2.2	NA	-4.3	-1.0	-1.7	-2.9*	-0.6	Triangular face, frontal bossing
5	<i>SHOX</i>	M	3200 (-0.4)	46 (-2.5)	7.5	4.0	-2.7	-	+0.2	-1.3 [#]	-1.9	
6	<i>ACAN</i>	M	3080 (-0.7)	46 (-2.5)	11.8	13.0	-2.0	+3.7	+1.0	-3.7*	0.9	
7	<i>PTPN11</i>	M	2680 (-1.6)	44 (-3.6)	12.3	8.0	-3.1	-	+0.3	-2.4	-1.4	
8	<i>NFI</i>	M	2480 (-2.1)	47 (-2.0)	7.3	2.8	-3.0	+0.8	-0.8	-2.0	NA	Two café au lait spots

M: male; F: female; SH: Sitting height:height ratio; BMI: body mass index; TH: Target height; SS: Short stature; NA: not available.

* - Presence of the mutation in heterozygous state, # - presence of mutation in mosaicism.

Figure 1: Strategy used to select children born small for gestational age (SGA) with isolated short stature of unknown cause included in this study. IUGR = Intrauterine growth restriction

Supplemental Table 1: Genes associated with growth disorders included in the targeted panel sequencing V1-2017.

IGFs/IGF1R pathway genes	IGF1 IGF1R ITGAV IGFBP3	IGF2 IGFBP1 IGFBP2 ITGB5	IGFBP4 IGFBP5 GRB10 IGFALS	IRS1 IRS2 AKT1	LZTR1 RAF1 BRAF	HRAS NRAS MAP2K1	MAPK1 SHOC2 CBL	RIT1 PIK3R1 SOS1	SOS2 NF1 MAP2K2	KRAS PIK3CA GRB2
Genes found at cGHarray/Exome	RAB31P DGCR8	GLRX3 SMAD4	GAB1 C16ORF55	IGF2BP2	ADIPOQ	CSNK2A1	CHD8	GALNS	MIR421	CTU2
Genes previously associated with growth disorders	A2ML1 AAAS ABCA1 ABCC8 ACADVL ACAN ACP5 ADAMTS10 ADAMTS17 AGL AGRP AIP AKT2 ALMS1 ALPL ANKRD11 ANOS1 APC CDON CDT1 APOB APOC2 APOC3 APPL1 AQP2 AR ARX ATM ATP1A1 ATRIP ATRX ATR AVPR2 BCL2 BMP1	BMP15 BMP2 BMP4 BMP8B BMPR1B BSC12 BTK CASR CBS CBX2 CDC6 CDC73 CDKN1A CDKN2B DMXL2 DNMT3A CENPJ CEP152 CEP19 CEP63 CHD7 CITED2 CLCN7 COL1A1 COL1A2 COL2A1 COL9A2 COL9A3 COMP CP CRIPT CUL7 CCDC8 CRTAP CTNNB1	CRTAP CTSK CUL3 CDKN1C DHCR7 DHH DICER1 DLK1 DMP1 DMRT1 G6PC GALNT3 DUSP6 DYRK1B EBF2 EIF2AK3 ENPP1 EPAS1 ESR1 EVC EVC2 EZH2 FAM111A FAM20C FBLN2 FBN1 FBN2 FEZ1 FGF17 FGF23 FGF8 FGF9 FGFR1 FGFR2 FGFR3	FIGLA FKBP10 FLNB FLRT3 FMR1 FOX12 FST IHH IL17RD GATA4 GATA6 GCG GCK GCM2 GDF5 GHR GHRH GHRHR GHRL GH1 GHSR GJA1 GLI2 GLI3 GLIS3 GLUD1 GNA11 GNAS GPC3 GPR101 GPR161 HDAC6 HDAC8 HMGA2 HESX1	HHIP HMGA1 HNF1B MC2R HNF4A MC4R PC HSD11B1 HSD11B2 IFITM5 MEST MCM9 MECP2 INS INSR IYD KCNJ11 KCNJ5 KDM6A KIT KITLG KL KLLN KMT2D/MLL2 KCNQ1OT1 LEP LEPR LHCGR LHX1 LHX3 LHX4 LHX8 LMNA LMNB2 LRP5 LTBP2 MAMLD1	MAP3K1 MATN3 MC2R MC4R PC PCSK1 MED12 MEF2C MEN1 MKRN3 MRAP MSX1 MTHFR NSUN2 NIPBL NIN NFIX NKX2-2 NNT NOTCH2 NPPB NPPC NPR2 NPR3 NR0B1 NR0B2 NR3C1 NR5A1 NSD1 NSMF ORC1 ORC4 ORC6 OSTM1 OTX2	OBSL1 PCNT P3H1 P4HB PAPP PAPPA2 PAPSS2 PAX4 PAX6 PAX8 ROR2 RNU4ATAC RECQL3/BLM RBBP8 RAD21 RUNX2 SRCAP PTPN11 PCYT1A PDGFRB PGR PHEX PITX2 PLAG1 PLAGL1 PLIN1 PNPLA6 POLR3A POLR3B POMC POR POU1F1 PPARG PPIB PRKG2 PROK2	PROKR2 PROP1 PTCH1 PTEN PTF1A PTH PTH1R PTHLH RAD51B RET RFX6 RNF216 RNPC3 SOX5 SOX6 SRCAP SMC1A SMC3 SMARCA4 SAMD9 SCARB1 SDHAF2 SEC24D SECISBP2 SEMA3A SEMA3E SERPINF1 SERPINH1 SHANK3 SHH SHOX2 SIM1 SIRT1 SIX3 SLC16A2	SLC19A2 SLC26A2 SLC26A4 SLC2A2 SLC34A1 SLC34A3 SLC37A4 SLC5A5 SMAD1 SOCS2 SOST SOX10 SOX2 SOX3 SOX8 SOX9 SP7 SPARC SPINK5 SPRY4 SQSTM1 STAG3 STAR STAT3 STAT5B STX16 TAC3 TACR3 TBCE TBX1 TCIRG1 TGFB3 TGIF1 THRA THRB	TIAL1 TMEM38B TNFRSF11A TNFRSF11B TP53 TPO TRIM37 TRPS1 TSHB TSHR DNA2 VDR VPS13B WDR11 WFS1 WNK1 WNK4 WNT1 WNT3A WNT4 WNT5A WT1 WWOX ZBTB20 ZFP57 ZMPSTE24 ZIC2

Supplemental Table 2: Criteria of pathogenicity of the *in silico* tools applied in our studies for exome analysis – 24-october-2018

Tool	Classification	Criteria used	Website
In Silico prediction of splice site alteration			
dbscSNV_ADA_SCORE and dbscSNV_RF_SCORE	Between 0 and 1	≥ 0.6	https://sites.google.com/site/jpopgen/dbNSFP
In Silico prediction of pathogenicity			
SIFT	T=Tolerated D=Deleterious	D	http://sift.jcvi.org/
POLYPHEN	B=Benign P=Probably pathogenic D=Deleterious.	P e D	http://genetics.bwh.harvard.edu/pph2/
Mutation Acessor	L=Low M=Moderate H=High.	M and H	http://mutationassessor.org/
Provean	N=Neutral D=Deleterious	D	http://provean.jcvi.org/genome_submit_2.php
CADD	Numeric	≥ 15	http://cadd.gs.washington.edu/
In Silico model for aminoacid conservation			
GERP	Numeric	≥ 2	http://mendel.stanford.edu/SidowLab/downloads/gerp/

DISCUSSÃO

Desordens do crescimento fazem parte de um grupo heterogêneo em relação às suas características fenotípicas, genéticas e moleculares. Apesar da avaliação clínica e laboratorial, o diagnóstico não é alcançado em cerca de 50 a 90% dos casos (49). Em relação aos pacientes com baixa estatura nascidos PIG, em aproximadamente 40%, nenhuma etiologia específica é identificada como a principal causa do comprometimento do crescimento (50).

Recentemente, com o advento do cariótipo molecular e do sequenciamento exômico, a abordagem genômica surgiu como uma estratégia importante para a investigação genética e para estabelecer a etiologia de muitos distúrbios do crescimento (7, 51). Além disso, estudos indicam que essa tecnologia parece ser mais rápida e barata do que a pesquisa tradicional (6, 31-33). Análise comparativa dos gastos da utilização do sequenciamento exômico na investigação de pacientes com suspeita de doença monogênica mostrou que essa metodologia foi capaz de triplicar a taxa de diagnóstico a um custo três vezes menor (31).

Neste estudo, nossa abordagem genômica utilizando o sequenciamento paralelo em larga escala permitiu o diagnóstico definitivo em 15% das crianças com baixa estatura isolada, e em 34% dos pacientes síndrômicos com baixa estatura nascidos PIG. Além disso, nossa estratégia genética, utilizando cariótipo molecular diagnosticou aproximadamente 15% dos pacientes com baixa estatura síndrômica de etiologia desconhecida. Outras análises também mostraram resultados semelhantes. Estudos que avaliaram crianças síndrômicas com baixa estatura por cariótipo molecular identificaram uma prevalência de 10% a 16% de variantes no número de cópias patogênicas ou provavelmente patogênicas (26, 40-42). Entre os estudos utilizando WES, esse número variou de 16,5 a 46% de acordo com a população analisada (6, 32, 33).

Apesar de não haver evidências bem estabelecidas sobre a abordagem padrão ouro para a avaliação genética de baixa estatura (52), nosso estudo mostrou que, no contexto de baixa estatura de causa desconhecida, a abordagem genômica pareceu ser uma ferramenta útil de investigação, especialmente entre crianças síndrômicas. Nesse grupo, mesmo após um histórico médico minucioso e um exame físico detalhado, a complexidade e multiplicidade das causas potenciais das síndromes que cursam com

baixa estatura dificultam o reconhecimento clínico. O distúrbio de crescimento é um componente de várias síndromes e pode ser devido a uma ampla variedade de mecanismos (15). Além disso, muitas síndromes são extremamente raras, apresentam espectro clínico variável e sobreposição de características físicas (6, 15, 33). Outro fator complicador na avaliação dessas crianças é a falta de profissionais com conhecimento em dismorfologia clínica. Segundo último levantamento da Sociedade Brasileira de Genética Médica, há apenas 241 médicos com título de especialista em genética médica, sendo essa a especialidade com menos médicos com título de especialista dentre as 53 especialidades avaliadas, o que dificulta ainda mais a avaliação especializada e o reconhecimento clínico dessas crianças (53).

Entre os pacientes com baixa estatura isolada de origem pré-natal, apesar da menor frequência de diagnósticos positivos, esta pesquisa genômica diagnosticou um em cada sete pacientes analisados. Considerando que todos esses pacientes foram avaliados seguindo os mais recentes consensos de investigação de pacientes com baixa estatura, baseados na tradicional avaliação global (exames laboratoriais e radiológicos) (44, 45) e estavam sem diagnóstico etiológico, podemos considerar 15% de diagnósticos uma taxa elevada de resultados positivos. Estudo que avaliou 235 pacientes com baixa estatura idiopática utilizando investigação tradicional, baseada em exames de imagem e laboratoriais, detectou apenas 1,3% das causas de baixa estatura (30), indicando que a abordagem genômica pode ser uma alternativa eficaz para um maior estabelecimento do diagnóstico etiológico de muitos pacientes com baixa estatura previamente denominados de “baixa estatura idiopática de origem pré-natal”.

Entre as variantes encontradas neste estudo, todos os genes foram previamente relatados como associados ao comprometimento do crescimento. No entanto, cada um deles afetou a altura de uma maneira diferente. Nos pacientes com baixa estatura isolada, genes associados à cartilagem de crescimento (*SHOX*, *ACAN*, *NPR2*, *IHH*) e à via RAS / MAPK (*NF1*, *PTPN11*) tiveram maior impacto nos resultados. As alterações encontradas nesse estudo, suportam o conceito que variantes alélicas em heterozigose em genes que afetam a cartilagem de crescimento são frequentemente presentes em pacientes com baixa estatura isolada. Por outro lado, entre os pacientes com condições sindrômicas encontramos defeitos envolvendo vários genes contíguos ou genes com efeitos pleiotrópico sobre o organismo. No nosso estudo, a maioria das variantes patogênicas encontradas estava relacionada a processos celulares fundamentais, vias de reparo de

DNA e vias intracelulares: *ACTG1*, *ANKRD11*, *AFF4*, *BCL11B*, *KIF11*, *KMT2A*, *POC1A*, *SRCAP*, *BRCA1* e *INPP5K*. E dentre os pacientes avaliados por cariótipo molecular, vimos que a maioria dos pacientes diagnosticados tinham deleções maiores que 1Mb, envolvendo vários genes, e muitas delas eram reincidentes em outras avaliações (22q11.21, 15q26, 1p36.33, Xp22.33, 17p13.3, 1q21.1, 2q24.2).

Outro aspecto identificado neste estudo, foi a importância da aferição do comprimento ao nascimento. Muitos dos pacientes diagnosticados tinham comprometimento do comprimento, independentemente de ser baixa estatura isolada ou sindrômica. É possível que no contexto de causas genéticas de distúrbios de crescimento de início pré-natal, ser PIG de comprimento represente uma característica relevante que favoreça a identificação de uma causa genética. Dentre os pacientes com achados dismórficos associados a baixa estatura, não conseguimos identificar nenhum fator relacionado a maior probabilidade de diagnóstico através da análise exômica. Dentre os pacientes avaliados por cariótipo molecular, essa análise estatística não foi realizada, entretanto, a grande maioria dos pacientes diagnosticados (~ 85%) tinha atraso de desenvolvimento neuropsicomotor e/ou déficit intelectual.

Nesse estudo, a avaliação fenotípica detalhada se mostrou muito importante na seleção dos pacientes para análise genômica. Durante o processo de seleção da casuística, o exame clínico detalhado, baseado na análise da dismorfologia, pôde estabelecer o diagnóstico em 45% dos pacientes e, dentre eles, pudemos confirmar a hipótese clínica inicial em 89% através de teste genético direcionado. Naturalmente, os pacientes reconhecidos clinicamente foram os casos sindrômicos mais comuns, com critérios diagnósticos mais bem estabelecidos, como síndrome de Silver-Russell (OMIM # 180860), acondroplasia (OMIM # 100800), hipocondroplasia (OMIM # 146000), discondrosteose de Leri-Weill (OMIM # 127300) e síndrome de Noonan (OMIM # 163950). Isso mostra que uma avaliação clínica cuidadosa e sistemática de pacientes com baixa estatura ainda é muito importante para estabelecer o diagnóstico clínico e selecionar o teste genético mais apropriado (33).

Diante disso, propomos o estabelecimento de um fluxograma de avaliação genética para pacientes com baixa estatura de causa não estabelecida com base em uma avaliação sistemática do fenótipo, visando testes genéticos alvo quando síndromes genéticas reconhecidas e, cariótipo molecular e/ou sequenciamento exômico, para pacientes sindrômicos não reconhecidos clinicamente. Pacientes com baixa estatura isolada, sem

causa identificada, deveriam ser avaliados por análise multigênica, por painel de baixa estatura e/ou sequenciamento exômico (Figura abaixo).

Figura: Proposta de fluxograma para investigação genética de crianças com baixa estatura de causa não estabelecida.

* Mudanças estruturais que têm consequências médicas, sociais ou estéticas significativas para o indivíduo afetado, e normalmente requerem intervenção médica (54).

A proposta desse fluxograma ocorre devido o aumento da complexidade e nos avanços dos métodos diagnósticos nos últimos anos, o que gera uma clara necessidade de revisão e atualização das diretrizes de investigação dos distúrbios de crescimento (10, 44-46). Um fluxo de investigação sistemática, baseado numa avaliação clínica especializada e exames genéticos bem indicados, com a utilização de cariótipo molecular e sequenciamento exômico nos pacientes com fenótipos extremos, não diagnosticados clinicamente e com alta probabilidade de ter uma base genética, poderia resultar em uma “jornada diagnóstica” mais curta para o paciente e a família, tornando a avaliação mais rápida e econômica.

Sabe-se ainda que o diagnóstico correto tem implicações importantes no, aconselhamento genético e na identificação de outros familiares (10). Em nosso estudo, a investigação de uma criança sindrômica de causa desconhecida por WES, permitiu o diagnóstico de Anemia de Fanconi devido uma variante patogênica no gene *BRCA1*. Isso favoreceu um aconselhamento genético preciso, desencadeou uma triagem precoce para

câncer tanto para a paciente quanto seus familiares e preveniu que se fosse prescrito tratamento com hormônio de crescimento (rhGH) para esta paciente. Outro benefício desse diagnóstico foi a consolidação de mutações no gene *BRCA1* como causa de Anemia de Fanconi, sendo o terceiro caso descrito na literatura (55). A publicação desse caso, favoreceu a descrição do gene no banco de dados do *Online Mendelian Inheritance in Man* (OMIM # 617883) em julho de 2018.

Ainda assim, nesse estudo, mesmo com abordagem genômica, muitos dos casos selecionados não foram diagnosticados. É provável que esses pacientes tenham modificações epigenéticas, mosaicismos somáticos, variações de sequência em regiões promotoras ou em outros locus regulatórios, áreas não examinadas por WES ou, alterações em regiões em que houveram falhas de cobertura. Além disso, é possível a existência de variações em mais de um gene responsável pelo fenótipo e variantes em genes que não pudemos comprovar a patogenicidade devido à ausência de estudos funcionais (17, 33).

Sabe-se que atualmente ainda existe uma grande dificuldade na análise das variantes identificadas. Entre os cerca de vinte mil genes no genoma humano, o banco de dados do OMIM sugere que apenas cerca de quatro mil genes estariam associados a doença, resultando em milhares genes desconhecidos ou genes associados a doenças não caracterizadas no genoma humano. E mesmo dentre os genes bem caracterizados, uma grande proporção de variantes é nova ou de significado clínico pouco claro (56). Nesse estudo, focamos apenas na identificação de variantes em genes já associados a distúrbios de crescimento. A avaliação de variantes de significado incerto (VUS) e possíveis genes candidatos não estava no escopo desta pesquisa, e o nosso termo de consentimento restringia apenas à reportagem de achados já bem associados ao diagnóstico na literatura.

Além disso, é provável que muitas crianças com baixa estatura leve possam ter herdado múltiplos polimorfismos, cada um deles responsável por um leve comprometimento do crescimento (57). E apesar da seleção de pacientes ter excluído fatores placentários e maternos conhecidos de retardo de crescimento intrauterino, também não podemos afastar, de forma absoluta, possíveis causas ambientais que possam ter comprometido o crescimento.

Ainda assim, apesar de todos os desafios que ainda existem para a implementação em maior escala do uso da tecnologia genômica na prática clínica, como custo, disponibilidade, familiaridade com o exame, e, mesmo, as próprias limitações dos testes,

como dificuldade de interpretação dos resultados e a otimização da aplicação do mesmo para os diferentes pacientes; nesse estudo, a utilização da abordagem genômica permitiu a identificação de diversas condições que não foram diagnosticadas através da “avaliação clínica tradicional”, seja pela raridade do diagnóstico e/ou pelo fenótipo não característico.

Espera-se que nos próximos anos, com o barateamento do exame, melhoria na sensibilidade e nas técnicas de análise, a abordagem genômica possa ser usada em maior escala. A implementação apropriada e antecipada dessa tecnologia poderia levar ao diagnóstico precoce e, possivelmente, identificar pacientes em estágios sintomáticos iniciais. Especialmente quando protocolos de acompanhamento ou tratamento específico estão disponíveis, sabe-se que a identificação precoce de distúrbios genéticos pode ter profundas implicações para os pacientes no que diz respeito ao controle da doença e à prevenção de complicações. Além disso, o uso em maior escala dessas tecnologias permitirá a identificação de novos genes associados à baixa estatura, resultando em uma melhor compreensão dos mecanismos de comprometimento do crescimento e, talvez, novas possibilidades de tratamento e/ou instituição de tratamento personalizado.

CONCLUSÕES

- A avaliação sistemática de crianças com baixa estatura, baseada na história clínica, no exame físico detalhado e avaliação genética através do sequenciamento exômico e cariótipo molecular são eficazes em estabelecer o diagnóstico etiológico em pacientes com baixa estatura de etiologia previamente desconhecida.

- A avaliação de um grupo de crianças com baixa estatura sindrômica através do cariótipo molecular identificou 15% dos casos como sendo portador de um desbalanço cromossômico patogênico (deleção ou duplicação).

- Detectamos sete microarranjos recorrentes (22q11, 15q26, Xp22.33, 1p36.33, 1q21.1, 17p13.3 e dissomias no cromossomo 14), responsáveis por até 40% dos desbalanços cromossômicos em crianças com baixa estatura sindrômica.

- O sequenciamento exômico estabeleceu o diagnóstico etiológico em 34% das crianças sindrômicas com baixa estatura de início pré-natal de causa desconhecida

- Estudo de um grupo de pacientes com baixa estatura isolada nascidos pequenos para idade gestacional utilizando sequenciamento paralelo em larga escala permitiu o estabelecer o diagnóstico etiológico de um em cada sete pacientes avaliados (15% dos pacientes).

ANEXO - Aprovação pelo Comitê de Ética em Pesquisa **PARECER CONSUBSTANCIADO DO CEP****DADOS DO PROJETO DE PESQUISA**

Título da Pesquisa: Causas genéticas de distúrbio de crescimento de início pré-natal

Pesquisador: Alexander Augusto de Lima Jorge

Área Temática:

Versão: 2

CAAE: 54415716.3.0000.0068

Instituição Proponente: HOSPITAL DAS CLINICAS DA FACULDADE DE MEDICINA DA U S P

Patrocinador Principal: HOSPITAL DAS CLINICAS DA FACULDADE DE MEDICINA DA U S P

DADOS DO PARECER

Número do Parecer: 1.645.329

Apresentação do Projeto:

Projeto já apresentado em parecer anterior

O tema do estudo é a avaliação das causas genéticas de distúrbio de crescimento de início pré-natal. Os autores pontuam que crianças nascidas pequenas para idade gestacional (PIG) constituem um grupo heterogêneo com quadros clínicos complexos, apresentando, além da baixa estatura, características dismórficas, malformações congênitas e/ou distúrbios de desenvolvimento neuro-psicomotor associadas. O mecanismo envolvido nesse processo decorre frequentemente de alterações genéticas, com diagnóstico muitas vezes não realizado, já que a disponibilidade e o custo de exames genéticos ainda são entraves consideráveis em nosso meio. No entanto, recentemente, com o advento de novas tecnologias de pesquisa gênica e a existência de programas de bioinformática, esse panorama vem sendo mudado, onde a disponibilidade de sites com informações genético-clínicas, como o Phenomizer e o Possum, se

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Continuação do Parecer: 1.645.329

tornaram

ferramentas úteis ao auxiliar o diagnóstico clínico dessas doenças. Assim, o estudo pretende realizar uma investigação clínica e genético-molecular de um grupo de pacientes nascidos pequenos para idade gestacional, analisando a acurácia de um protocolo de avaliação clínica e desses programas utilizados para identificação de síndromes genéticas (Phenomizer e Possum) na identificação da causa etiológica de crianças nascidas PIG, desenvolvendo estratégias para o diagnóstico etiológico do distúrbio de crescimento destas crianças combinando dados clínicos, laboratoriais, radiológicos e de investigação genético-molecular. Para esse fim, 150 pacientes com histórico de terem nascido pequenos em peso e/ou comprimento para idade gestacional e não terem apresentado recuperação espontânea pós-natal até os 2 anos de idade serão submetidos à investigação clínica, de imagem e laboratorial. Para a genotipagem, amostras de DNA serão obtidas a partir de leucócitos de sangue periférico ou de células extraídas por swab da mucosa oral dos indivíduos arrolados na pesquisa. Através dos programas Phenomizer e POSSUM, as características encontradas nos pacientes serão plotadas de forma que sejam sugeridos possíveis diagnósticos etiológicos.

Objetivo da Pesquisa:

O objetivo principal é realizar investigação clínica e molecular prospectiva de uma coorte de crianças nascidas PIG e sem recuperação espontânea na vida pós-natal buscando a identificação de causas genéticas. Os objetivos específicos são analisar a acurácia de um protocolo de avaliação clínica e de programas utilizados para identificação de síndromes genéticas (Phenomizer e Possum) na identificação da causa etiológica de crianças nascidas PIG e desenvolver uma estratégia para o diagnóstico etiológico do distúrbio de crescimento destas crianças combinando dados clínicos, laboratoriais, radiológicos e de investigação genético-molecular.

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Avaliação dos Riscos e Benefícios:

Os autores relatam apenas o desconforto associado à coleta de sangue e o benefício será a identificação de possíveis defeitos em genes, responsáveis pelo aparecimento da doença que gerou as alterações do crescimento ou desenvolvimento, podendo, a partir dessas informações, realizar-se um aconselhamento genético no futuro.

Comentários e Considerações sobre a Pesquisa:

Nenhuma.

Considerações sobre os Termos de apresentação obrigatória:

Apresentado novo TCLE agora adequado

Recomendações:

Aprovação.

Conclusões ou Pendências e Lista de Inadequações:

Aprovação.

Considerações Finais a critério do CEP:

Em conformidade com a Resolução CNS nº 466/12 – cabe ao pesquisador: a) desenvolver o projeto conforme delineado; b) elaborar e apresentar relatórios parciais e final; c) apresentar dados solicitados pelo CEP, a qualquer momento; d) manter em arquivo sob sua guarda, por 5 anos da pesquisa, contendo fichas individuais e todos os demais documentos recomendados pelo CEP; e) encaminhar os resultados para publicação, com os devidos créditos aos pesquisadores associados e ao pessoal técnico participante do projeto; f) justificar perante ao CEP interrupção do projeto ou a não publicação dos resultados.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_644957.pdf	02/06/2016 13:01:42		Aceito
TCLE / Termos de Assentimento /	TCLE.docx	02/06/2016 13:01:22	Thais Kataoka Homma	Aceito

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Justificativa de Ausência	TCLE.docx	02/06/2016 13:01:22	Thaís Kataoka Homma	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_PIG.docx	21/03/2016 12:18:56	Thaís Kataoka Homma	Aceito
Outros	cappesq.pdf	21/03/2016 12:07:05	Thaís Kataoka Homma	Aceito
Folha de Rosto	Folha_de_rosto.pdf	21/03/2016 10:30:34	Thaís Kataoka Homma	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

SAO PAULO, 22 de Julho de 2016

Assinado por:
ALFREDO JOSE MANSUR
(Coordenador)

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