RESEARCH

Genetic diagnosis of congenital hypopituitarism by a target gene panel: novel pathogenic variants in *GLI2*, *OTX2* and *GHRHR*

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Abstract

Aim: Congenital hypopituitarism has an incidence of 1:3500–10,000 births and is defined by the impaired production of pituitary hormones. Early diagnosis has an impact on management and genetic counselling. The clinical and genetic heterogeneity of hypopituitarism poses difficulties to select the order of genes to analyse. The objective of our study is to screen hypopituitarism genes (candidate and previously related genes) simultaneously using a target gene panel in patients with congenital hypopituitarism.
Methods: Screening of 117 subjects with congenital hypopituitarism for pathogenic variants in 26 genes associated with congenital hypopituitarism by massively parallel sequencing using a customized target gene panel.
Results: We found three novel pathogenic variants in *OTX2* c.295C>T:p.Gln99*, *GLI2* c.1681G>T:p.Glu561* and *GHRHR* c.820_821insC:p.Asp274Alafs*113, and the previously reported variants in *GHRHR* c.57+1G>A and *PROP1* [c.301_302delAG];[c.109+1G>A].
Conclusions: Our results indicate that a custom-designed panel is an efficient method to screen simultaneously variants of biological and clinical relevance for congenital GH deficiency. A genetic diagnosis was possible in 5 out of 117 (4%) patients of our cohort. We identified three novel pathogenic variants in *GHRHR*, *OTX2* and *GLI2* expanding the spectrum of variants associated with congenital hypopituitarism.

Key Words
- congenital hypopituitarism
- growth hormone deficiency
- mutations
- massively parallel sequencing
- high-throughput nucleotide sequencing
- target gene panel

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Introduction

Congenital hypopituitarism (CH) is a rare disorder (incidence of 1:3500–10,000 births) defined by the deficiency of one or more pituitary hormones (1, 2). Clinical presentation varies, ranging from isolated growth hormone deficiency (IGHD) to combined pituitary hormone deficiencies (CPHD). CH also varies with respect to hypothalamic–pituitary anatomy from normal magnetic resonance imaging to the presence of midline defects such as pituitary stalk interruption syndrome (PSIS) and/or severe complex

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craniofacial malformations, such as septo-optic dysplasia (SOD).

The genetic defects leading to CH were classified in: defects in GH secretion (*GH1*, *GHRHR*); defects in pituitary cell differentiation (*PROP1*, *POU1F1*) and defects in pituitary development (such as *HESX1*, *GLI2*, *OTX2*) (2). The most common genes implicated are those encoding *GH1* and *GHRHR* in IGHD and *PROP1* in CPHD, especially in certain geographical regions. Until now, most studies used the candidate-gene approach as the main strategy to identify the genetic cause of CH (3). As this strategy considers the patient's clinical presentation to guide the molecular investigation, genes were heterogeneously screened in most studies – the same genes were not analysed in the entire cohort. Moreover, as the clinical phenotype is highly variable, we cannot assure that all possible causative genes were excluded (4). The use of massive parallel sequencing, mostly targeted sequencing panel or whole exome sequence (WES), allows the screening of several genes simultaneously with high accuracy in a less time-consuming way.

In the present study, we developed a comprehensive gene panel for systematic assessment of CH including previously known and newly discovered genes related to CH in a large cohort with the objective of expanding the clinical and etiological spectrum of this disease.

Patients and methods

Patients with CH followed at the Unidade de Endocrinologia do Desenvolvimento (São Paulo, Brazil) and Hospital de Niños Santísima Trinidad (Cordoba, Argentina) with previous positive genetic diagnosis by Sanger sequencing with the candidate-gene approach were not included in this study. We selected 117 subjects with CH of unknown cause (104 from Brazil and 13 from Argentina) (Fig. 1). The patients were predominantly male (55%, $n=64$), only eight were born from consanguineous parents and another 25 had relatives with short stature. Most of them had CPHD (77%, $n=90$) and ectopic posterior pituitary (EPP) lobe (68%, $n=79$) (Fig. 1). The median age of the first visit was 11 years (ranging from 6 days to 51.9 years), height SDS of –3.8 (ranging from –1.8 to –7.4) and GH peak of 1.7 µg/dL (ranging from undetectable to 4.7 µg/dL). This study was approved by the respective Local Ethics Committees ('Comitê de Ética para Análise de Projetos de Pesquisa' in Brazil and 'Comité Institucional de Ética de la Investigación en Salud del Niño y del Adulto' in Argentina) and the patients or guardians gave their written informed consent.

Serum levels of TSH, GH, LH, FSH, PRL, cortisol, dehydroepiandrosterone sulphate, total thyroxine, free thyroxine, insulin-like growth factor 1 (IGF-1), oestradiol or testosterone levels were measured at baseline. Stimulatory tests were performed for the diagnosis of GH and other pituitary hormone deficiencies as previously described (5). In neonates, GH (cut-off limit <5 ng/mL) deficiency was evaluated considering the baseline GH measurement during hypoglycaemia and IGF-1 and IGFBP-3 levels less than –2 SDS for age and sex. As patients were studied at different centres using different hormonal assays, normal ranges of each centre were considered.

Target gene panel

Genomic DNA was extracted from peripheral blood leucocytes of all patients. A customized gene panel was

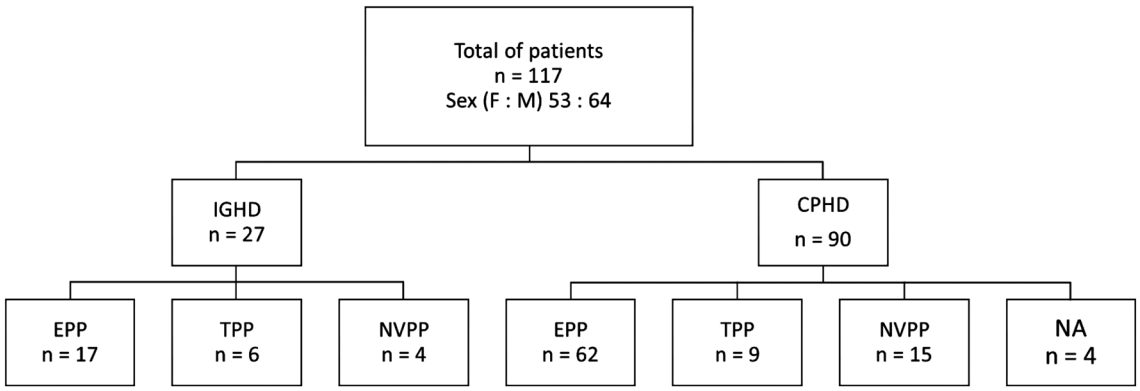

Figure 1
Clinical features of sequenced patients. CPHD, combined pituitary hormone deficiency; EPP, ectopic posterior pituitary lobe; IGHD, isolated growth hormone deficiency; NA, not available; NVPP, non-visualized posterior pituitary; TPP, topic posterior pituitary lobe.

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designed using Agilent Sure Design (Agilent Technologies, Inc.) with probes for 26 genes previously related to hypopituitarism *GH1, GHRH, GHRHR, GHSR, PROP1, POU1F1, GLI2, HESX1, LHX3, LHX4, OTX2, PITX2, ARNT2, DMXL2, FGF8, FGFR1, GPR161, HHIP, IGSF1, KAL1, PROKR2, RNPC3, SHH, SOX2, SOX3* and *TGIF1* (2).

Genomic DNA was mechanically fragmented using Covaris. Libraries were constructed using SureSelect Target Enrichment System Technology in accordance with the manufacture's protocols (Agilent Technologies). The sequences were generated in the Illumina NextSeq 500 platform running on paired-end mode. Reads were aligned to the GRCh37/hg19 assembly of the human genome with the Burrows-Wheeler aligner (BWA-MEM) (GNU General Public License version 3.0 (GPLv3), MIT License, Cambridge, MA, USA). Variant calling was performed with Freebayes (https://wiki.gacrc.uga.edu/wiki/Freebayes) and the resulting variant call formats (VCFs) were annotated with ANNOVAR (http://annovar.openbioinformatics.org/en/latest/).

The targeted panel sequencing data were screened for rare variants (MAF <1%) in public global and Brazilian databases: gnomAD (http://gnomad.broadinstitute.org/) and ABraOM (http://abraom.ib.usp.br/), located in exonic regions and consensus splice site sequences. Next, our variant filtration prioritized genes based on their potential to be pathogenic: loss-of-function (LoF) variants and variants predicted to be pathogenic by multiple in silico programmes (SIFT, PolyPhen2, Mutation Taster, PROVEAN and CAAD). The sequencing reads carrying candidate variants were inspected visually using the Integrative Genomics Viewer (IGV) to reduce false-positive calls. Sanger sequencing confirmed all pathogenic and probably pathogenic variants. (Primer sequence and amplification protocols are available upon request.) The variants at the final list were assessed for the clinical interpretation of pathogenicity using InterVar (http://wintervar.wglab.org/) according to the American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) variant pathogenicity guidelines (6).

Results

Molecular results

The median coverage depth of the coding regions in our panel data was 462×, with at least 99.6% of the sequenced bases covering more than 20-fold.

We identified causative pathogenic variants in 5 of 117 CH patients (diagnostic yield of 4%) (Table 1). These variants were in genes directly involved in GH secretion (*GHRHR*), in pituitary cell differentiation (*PROP1*) and in pituitary development (*GLI2*, *OTX2*). All variants were absent or extremely rare in local and public databases (Table 1).

Biallelic LoF variants were identified in *GHRHR* and *PROP1* genes. One pathogenic mutation located in a consensus splice site (c.57+1G>A) and another causing a frameshift mutation (c.820_821insC:p.Asp274Alafs*113) in *GHRHR* were found in homozygous state, each in one patient with IGHD. Two pathogenic variants in *PROP1* were identified in compound heterozygous state in one patient with CPHD by this panel and has been recently published with a cohort of *PROP1* Brazilian patients (7).

Two novel heterozygous nonsense pathogenic variants were identified in *GLI2* (c.1681G>T:p.Glu561*) and *OTX2* (c.295C>T:p.Gln99*), each one in one subject.

Phenotype of subjects with variants of interest

Characteristics of affected subjects are detailed in Table 2. Weight, length at birth, GH peak, age and height SDS at first visit did not differ significantly from those with negative molecular results. All patients were severely affected at presentation (median height SDS of −5.8, ranging from −7.8 to −4.4) (Table 2).

Among the patients with homozygous variants in *GHRHR*, one patient was born to consanguineous parents and the other patient's parents came from the same small village and probably presented some degree of consanguinity. The second case had a short brother and the presence of the same variant was identified in homozygous state by Sanger sequencing.

The patients carrying variants in *GHRHR* had IGHD and topic posterior pituitary (TPP). The patient with *PROP1* mutation had CPHD (GH, TSH and partial ACTH deficiencies) and TPP.

Patients with pathogenic variant in genes precociously expressed during hypothalamic-pituitary development, *GLI2* and *OTX2*, presented IGHD and EPP.

The mother of the patient with *GLI2* variant, also carrying the same mutation, has short stature (−4.0 HSDS), EPP, hypoplasia of anterior pituitary and thin stalk, despite having IGF-1 levels in the normal range for her advanced age. The index patient's mother referred that her brother and nephew (not available for clinical or genetic studies) had polydactyly.

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Table 1 Pathogenic variants identified by targeted panel sequencing in a cohort of 117 patients with congenital hypopituitarism.

Patient	Gene	Allelic variant	GnomAD	ABraOM	Inheritance	Evidence of pathogenicity according to ACMG/AMP	Final ACMG/AMP classification	RefSeq ID
1	*GHRHR*	c.57+1G>A	0.00001957	0	Homozygous	PVS1, PM2, PP3	Pathogenic	NM_000823.3 rs2302022
2	*GHRHR*	c.820_821insC: p.Asp274Alafs*113	0	0	Homozygous	PVS1, PM2, PP3	Pathogenic	NM_000823.3
3	*PROP1*	c.301_302del: p.Leu102Cysfs*8	0,0001805	0	Compound Heterozygous	PVS1, PM1, PP5	Pathogenic	NM_006261.4 rs193922688
		c.109+1G>A	0	0	Compound Heterozygous	PVS1, PM2, PP3	Pathogenic	NM_006261.4
4	*GLI2*	c.1681G>T:p.Glu561*	0	0	Heterozygous	PVS1, PM2, PP3	Pathogenic	NM_005270.4
5	*OTX2*	c.295C>T:p.Gln99*	0	0	Heterozygous	PSV1, PM2, PP3	Pathogenic	NM_172337.2

PVS1, null variant (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multiexon deletion) in a gene where LoF is a known mechanism of disease; PM1, located in a mutational hot spot and/or critical and well-established functional domain (e.g. active site of an enzyme) without benign variation; PM2, absent from controls (or at extremely low frequency if recessive) in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium; PP3, multiple lines of computational evidence support a deleterious effect on the gene product; PP5, reputable source recently reports variant as pathogenic, but the evidence is not available to the laboratory to perform an independent evaluation.

The patient carrying the mutation in *OTX2* had microphthalmia, nystagmus and neuropsychomotor developmental delay.

Discussion

In this study, we screened simultaneously 26 genes in a cohort of 117 patients with CH using a target gene panel approach and identified five causative pathogenic variants (positive yield of 4%) in genes *GHRHR*, *PROP1*, *GLI2* and *OTX2*. A limitation of this study is that this cohort included, in addition to patients who were not sequenced before, patients in whom some candidate genes had been negative by Sanger sequencing. Therefore, a higher yield might be expected in a prospective cohort.

We found two null homozygous mutations in *GHRHR* in two patients and, as expected, both patients had IGHD with orthotopic posterior pituitary lobe. One variant was a frameshift insertion described for the first time in the present study and the other was a splicing mutation (c.57+1G>A) initially reported in a large kindred with isolated GH deficiency from Itabaianinha in Northeastern Brazil (8). The same variant in homozygous and compound heterozygous state was also previously described in other Brazilian familial and sporadic cases and a founder effect was demonstrated (9). Most patients with autosomal recessive IGHD have variants in *GHRHR*

Table 2 Clinical characteristics of patients with pathogenic variants.

Patients	Gene	Sex	Delivery	Family history	Height SDS at first visit	Hormonal deficiency	MRI	Associated complex phenotype
1[a]	*GHRHR*	F	Caesarean section	No	−5.2	IGHD	Normal	Deafness
2[b]	*GHRHR*	F	Normal	Affected brother	−5.8	IGHD	Anterior pituitary hypoplasia, NVPP	Absent
3	*PROP1*	M	Normal	No	−7.8	GH, TSH, ACTHp	Anterior pituitary hypoplasia, TPP	Absent
4	*GLI2*	M	Caesarean section, hemorrhage during labour	Reported short stature	−4.4	IGHD	EPP	Absent
5	*OTX2*	M	Normal	No	−6.1	IGHD	EPP, septo-optic dysplasia	Microphthalmia, nystagmus, neuropsychomotor developmental delay

[a]Born to consanguineous healthy parents; [b]parents came from the same small village.
EPP, ectopic posterior pituitary; IGHD, isolated growth hormone deficiency; MRI, magnetic resonance imaging; NVPP, non visualized posterior pituitary; TPP, topic posterior pituitary.

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which is therefore one of the first candidate genes to be screened in consanguineous cases (10).

We found two pathogenic variants in compound heterozygous state in *PROP1* [c.301_302delAG];[c.109+1G>A] in one subject. Whereas c.301_302delAG is the most common *PROP1* variant worldwide due to a founder effect, the c.109+1G>A variant was first found by this panel and recently published as a novel variant by our Unit together within a cohort of Brazilian patients (7, 11). Although there is an increased prevalence of familial cases and consanguinity among patients with CPHD due to *PROP1* variants, the patient described here is a sporadic case and the only affected member of his family (7).

One patient in our cohort had a pathogenic stop codon variant in *GLI2* (p.Glu561*) that predicts a truncated protein with loss of the zinc finger and transactivation domains. Variants in *GLI2* have incomplete penetrance and can lead to complex midline defects, holoprosencephaly (HPE), cleft palate, polydactyly and CPHD with variable phenotypes (12, 13). Our patient presents a mild phenotype with only IGHD, and no craniofacial defects.

A novel nonsense variant was identified in *OTX2* (p.Gln107*) gene in heterozygous state, predicting a truncated protein with the loss of 642 amino acids. Variants in *OTX2* were associated to severe ocular malformations such as anophthalmia, microphthalmia and variable degrees of hypopituitarism. Most patients with *OTX2* mutations also exhibit brain anomalies and/or seizures that are not present in our case (14).

Until now, only one study used a target gene panel for the diagnosis of a cohort of patients with CH: 51 patients from 44 independent pedigrees were evaluated for mutations using a target sequence panel, in which genes were captured using smMIPS (single-molecule molecular inversion probe capture assay). In two families with IGHD phenotype, the same heterozygous mutation in *GH1* gene (p.Arg209His) was identified (15).

Other studies used target panel or whole exome sequencing to study patients with short stature due to different etiologies including few patients with GHD. Dauber *et al*. sequenced 1077 genes in 192 patients (31 with CPHD) and found four pathogenic variants in *PTPN11* and *IGF1R* genes (16). Hauer *et al*. sequenced 200 patients with short stature who underwent extensive prior endocrinological and diagnostic workup to exclude defects of the growth hormone pathway and identified one variant in the *GHSR* gene (17). At last, another study performed exome-sequencing on ten patients with CPHD and their unaffected parents and suggested *SLC20A1* and *SLC15A4* as new candidate genes (18).

Using a target gene panel in large cohorts allows the screening of many genes simultaneously with higher accuracy avoiding the misinterpretation caused by the lack of phenotype-genotype correlation. Therefore, we believe that this approach has advantages for the genetic diagnosis when compared to the candidate-gene approach.

The diagnosis of GH deficiency is complex and usually involves auxological, hormonal and imaging studies (19). If recent advances in genetic studies will curtail this diagnostic odyssey remains to be elucidated. The modest positive rate of genetic screening of CH (4%: this study) obtained with gene panels indicates that this genetic tool still cannot replace the clinical diagnosis of CH.

We conclude that a custom-designed target gene panel is an efficient method to screen simultaneously variants of biological and clinical relevance for congenital GH deficiency. A genetic diagnosis was possible in 5 out of 117 (4%) patients of our cohort. We identified novel variants in *GHRHR*, *PROP1*, *OTX2* and *GLI2*. Further studies are necessary to understand the participation of other genetic, epigenetic phenomena and/or environmental factors in the aetiology of most patients with CH.

Declaration of interest

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

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