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Next generation sequencing panel based on single molecule molecular inversion probes for detecting genetic variants in children with hypopituitarism

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Abstract

Background: Congenital Hypopituitarism is caused by genetic and environmental factors. Over 30 genes have been implicated in isolated and/or combined pituitary hormone deficiency. The etiology remains unknown for up to 80% of the patients, but most cases have been analyzed by limited candidate gene screening. Mutations in the *PROP1* gene are the most common known cause, and the frequency of mutations in this gene varies greatly by ethnicity. We designed a custom array to assess the frequency of mutations in known hypopituitarism genes and new candidates, using single molecule molecular inversion probes sequencing (smMIPS).

Methods: We used this panel for the first systematic screening for causes of hypopituitarism in children. Molecular inversion probes were designed to capture 693 coding exons of 30 known genes and 37 candidate genes. We captured genomic DNA from 51 pediatric patients with CPHD (n=43) or isolated GH deficiency (IGHD) (n=8) and their parents and conducted next generation sequencing.
Results: We obtained deep coverage over targeted regions and demonstrated accurate variant detection by comparison to whole-genome sequencing in a control individual. We found a dominant mutation *GH1*, p.R209H, in a three-generation pedigree with IGHD.
Conclusions: smMIPS is an efficient and inexpensive method to detect mutations in patients with hypopituitarism, drastically limiting the need for screening individual genes by Sanger sequencing.

Keywords
Single molecule Molecular Inversion Probes, Growth Hormone Deficiency, Congenital Hypopituitarism, GH1

Introduction

Pituitary dysfunction is an important human health problem that is caused primarily by congenital birth defects and pituitary adenomas. Hormone deficiencies can be isolated, and **I**solated **g**rowth **h**ormone **d**eficiency (**IGHD**) is the most common, or involve two or more pituitary hormones: **c**ombined **p**ituitary **h**ormone **d**eficiency (**CPHD**). IGHD progresses to CPHD in 45% of patients (Blum et al., 2014; Otto et al., 2015). Only 16% of the cases of congenital CPHD can be explained by mutations in known genes (Cogan et al., 1998; Coya et al., 2007; Dateki et al., 2010; de Graaff et al., 2010; De Rienzo et al., 2015; Deladoey et al., 1999; Diaczok, Romero, Zunich, Marshall, & Radovick, 2008; Dusatkova et al., 2016; O. V. Fofanova et al., 1998; Halasz et al., 2006; Kandemir et al., 2012; Kim et al., 2003; Lebl et al., 2005; Lemos et al., 2006; McLennan et al., 2003; Mehta & Dattani, 2008; Navardauskaite et al., 2014; Pfaeffle et al., 2007; Rainbow et al., 2005; Reynaud et al., 2006; Takagi et al., 2012; Turton, Mehta, et al., 2005; Vieira, Boldarine, & Abucham, 2007), and for IGHD the rate is about 11% (Alatzoglou & Dattani, 2010; Wit et al., 2016). Molecular diagnosis is critical for predicting disease progression and risk of recurrence (Agarwal, Bhatia, Cook, & Thomas, 2000; Bottner et al., 2004; Fluck et al., 1998; Pernasetti et al., 2000). Some congenital cases of CPHD are

associated with enlarged pituitary glands, and molecular diagnosis distinguishes these as benign and distinct from adenomas that appear similar on MRI, avoiding unnecessary intracranial surgery (Mendonca et al., 1999; Riepe et al., 2001). Unidentified hypopituitarism can result in infant death, and some types of hypopituitarism are progressive, leading to life-threatening disorders secondary to hypoglycemia and adrenal insufficiency (30-33).

Congenital combined pituitary hormone deficiency (CPHD) arises from defects in pituitary development and is sometimes associated with extra pituitary abnormalities, such as cleft lip/palate, a short stiff neck, and hypoplastic optic nerves. For example, mutations in *HESX1* (OMIM reference number *601802) can cause septo-optic dysplasia (SOD), CPHD, and IGHD (Dasen et al., 2001; Dattani et al., 1998; Gage et al., 1996) , mutations in *OTX2* (*600037) can cause craniofacial abnormalities, including anophthalmia with or without IGHD or CPHD (Dateki et al., 2008; Diaczok et al., 2008; Matsuo, Kuratani, Kimura, Takeda, & Aizawa, 1995; Mortensen, MacDonald, Ghosh, & Camper, 2011; Mortensen, Schade, Lamonerie, & Camper, 2015; Nishida et al., 2003; Tajima et al., 2008), and mutations in *GLI2* (*165230) can cause holoprosencephaly, CPHD, or hypogonadism hypogonadotropic (HH) (Arnhold, Franca, Carvalho, Mendonca, & Jorge, 2015; Flemming et al., 2013; Franca et al., 2010). Mutations in *PROP1* (*601538) are the most common known cause of CPHD, accounting for 11% of total cases worldwide (Cogan et al., 1998; Deladoey et al., 1999; O. Fofanova et al., 2000; Rosenbloom et al., 1999; Wu et al., 1998). *Prop1* is the first pituitary-specific gene in the transcriptional hierarchy of genes that cause CPHD, and it is essential for developing a normal stem cell pool and for stimulating stem cells to undergo an epithelial to mesenchymal transition-like (EMT) process necessary for cell migration and differentiation (Perez Millan, Brinkmeier, Mortensen, & Camper, 2016). *Prop1* is necessary to activate expression of *Pou1f1* (*173110) (Sornson et al., 1996), and *POU1F1* is mutated in individuals with CPHD or IGHD (Radovick et al., 1992; Sobrier et al., 2016; Tatsumi et al., 1992; Turton, Reynaud, et al., 2005; Turton, Strom, Langham, Dattani, & Le Tissier, 2012) and no other clinical features. From these examples, it is clear that CPHD is part of a spectrum disorder that spans from severe abnormalities including holoprosencephaly (HPE) and septo-optic dysplasia (SOD) to milder cases with hypogonadotropic hypogonadism or IGHD (Fang et al., 2016; Raivio et al., 2012). The most common genes implicated in IGHD are those encoding growth hormone (*GH1)* (*139250) and the growth hormone releasing hormone receptor (*GHRHR*) (*139191). Also, IGHD is sometimes caused by mutations in genes involved in early embryonic development, like OTX2, HESX1, SOX2 (*184429) and SOX3 (*313430) (Alatzoglou et al., 2009; Kelberman et al., 2006).

The identification of genetic mutations is important for understanding the variability and progression of the disease, and as a foundation for the development of new treatments. Until recently, genetic testing was performed on a gene-by-gene basis, starting with the most likely candidate gene. With the incorporation of next generation sequencing technologies, it is now possible to test a large number of genes from several individuals in a single assay, reducing effort, costs and time. Here, we present a novel and cost-effective approach to screen for coding mutations in known and suspected CPHD and IGHD risk genes, based upon single-molecule molecular inversion probe sequencing (smMIPS) (Hiatt, Pritchard, Salipante, O'Roak, & Shendure, 2013). We established a panel of 67 genes associated with CPHD and IGHD in humans and mice, including new candidate genes found by analysis of *Prop1* mutant mice (Perez Millan et al., 2016). This panel targets 693 coding exons. We analyzed 51 pediatric patients from Argentina with CPHD or IGHD and their parents. We found a dominant mutation p.R209H in *GH1* in a three-generation pedigree with isolated growth hormone deficiency type II. Using single molecule molecular inversion probes capture and deep sequencing is an efficient and inexpensive method to detect mutations in patients with hypopituitarism. Identifying these potential variants will make it feasible to predict clinical outcomes from genetic data, which is necessary for patient diagnosis and prognosis, and for assessing the risk of future affected individuals.

Materials and Methods

Subjects

Whole blood was collected from 51 Argentinean patients belonging to 44 unrelated families diagnosed with IGHD or CPHD at the Hospital de Niños Ricardo Gutiérrez, Buenos Aires, Argentina. Samples were collected from unaffected parents and other relatives when feasible and warranted. All subjects were informed of the purpose of the study and their written consent was obtained. Parental consent was sought for patients under the age of 18. The study was approved by the Ethics Committee of Hospital de Niños Ricardo Gutiérrez, Buenos Aires, Argentina. The University of Michigan Institutional Review Board approved the use of anonymized DNA samples.

Patients were diagnosed with growth hormone deficiency (GHD) on the basis of abnormally low growth velocity and peak GH less than 4.8 µg/L after sequential arginine/clonidine pharmacological stimulation tests. Thyroid-stimulating hormone (TSH) deficiency was diagnosed in individuals with free thyroxine <1.0 ng/dl with low or normal TSH levels. Low TSH is ≤ 10 mU/l in patients under 2 months of age and ≤ 6.5 mU/l in older infants;

ACTH deficiency was diagnosed based on low basal serum cortisol, <30.3 nmol/L in patients under 2 months of age, <58 nmol/L in patients between 2 and 6 months, and <165 nmol/L in older infants (Ballerini et al., 2010). Individuals with low or normal plasma ACTH were considered affected if serum cortisol was <550 nmol/L under hypoglycemia. Prolactin deficiency was considered in individuals with serum levels <2.5th centile for sex and age. Central diabetes insipidus was diagnosed when polyuria was associated with a urinary:plasma osmolarity ratio of <1.5 and the patient had a plasma osmolality >300 mosm/l. Gonadotropin deficiency was diagnosed in boys aged between 15 days and 6 months when serum luteinizing hormone (LH) and testosterone (T) were <5th centile, <0.8 IU/l and <30 ng/dl, respectively. In girls from the age of 15 days to 2 years, gonadotropin deficiency was assumed when follicle-stimulating hormone (FSH) levels were <1.0 IU/l (Braslavsky et al., 2015). In older patients, gonadotropin deficiency was defined as delayed or absent pubertal development with a low serum testosterone (T < 3.47 nmol/L) associated with inappropriately low or normal LH and FSH levels. CPHD was defined as the presence of hormone deficiency affecting at least two anterior pituitary hormone-producing cell types. Brain and Pituitary Magnetic Resonance Imaging (MRI) was performed in all patients.

Genomic DNA isolation

Genomic DNA was extracted from peripheral blood cells using Puregene Blood kit (QIAGEN) according to the protocol provided by the manufacturer. The DNA was quantified using QuantiFluor® dsDNA System (Promega) and the DNA concentration was normalized to 25ng/µl for smMIPS assay. The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of DNA. All DNA samples included in the panel have a 260/280 ratio between 1.8 and 2.1. To assess smMIPS accuracy, we included DNA from GM12878, a gold-standard reference cell line, with publically available variant calls from deep whole genome sequencing (WGS) (Zook et al., 2014) (Coriell Institute for Medical Research, Camden, NJ).

Molecular Inversion Probes design, capture and Sequencing

67 genes were included in the smMIPS panel, to target 693 coding exons totaling 174.1 kb of coding sequence (File S1). This panel was designed targeting the coding exons (as defined by the UCSC Genome Browser, “Known Gene” table, hg19 build), padded by ≥ 25 bp in each direction to include exon-intron boundaries. Design, preparation, and capture using smMIPS probes were performed as previously described (Yoon et al., 2015). Briefly, a library of smMIPS probes was designed for batch synthesis using custom python scripts. Probe

sequences were synthesized on a single microarray as 150mers by CustomArray, Inc. smMIPS probes were PCR amplified from the resulting pool using externally directed primers "mipPrep1F" and "mipPrep1R" (5'-GGTAGCAAAGTGCAGATGTGCTCTTC-3', and 5'-TGAACTCACACTGCTCTGAACTCTTC-3'), digested overnight with EarI (NEB) to remove flanking amplification primers, purified with one volume SPRI beads supplemented with five volumes isopropanol, and eluted in Tris-EDTA pH 8. For smMIPS captures, approximately 3 ng smMIPS probes were combined with 125 ng genomic DNA, in a reaction mixture including Ampligase DNA Ligase Buffer 1X (Epicentre), 0.4 uM dNTPs (NEB), 3.2U HemoKlentaq (NEB) and 1U Ampligase (Epicentre). After denaturation at 95C for 10 minutes and incubation at 60C for 20 hours, linear probes and the remaining genomic DNA were removed by exonuclease treatment with ExoI and ExoII (NEB). The captured material was amplified by PCR using barcoded primers. The resulting PCR products were pooled (120 samples) for one lane of paired-end 100 bp sequencing on an Illumina HiSeq 2500 instrument at the University of Michigan Sequencing Core.

Data analysis pipeline

We used a freely-available, open source pipeline for smMIPS-specific aspects of sequence alignment, downstream processing, and quality control (available at https://github.com/kitzmanlab/mimips). Briefly, this pipeline uses bwa-mem (Li, 2013) to align reads to the human reference genome (build GRCh37), followed by custom python scripts to remove sequences derived from smMIPS probe oligonucleotides, and to remove reads with duplicate molecular tags. Variant calling was performed with Haplotype Caller from the Genome Analysis Toolkit (GATK) (McKenna et al., 2010) (DePristo et al., 2011) (Van der Auwera et al., 2013). The resulting VCF was further annotated with SnpEff/SnpSift (Cingolani et al., 2012) using the following main sources dbSNP, ExAC (Karczewski et al., 2017), ClinVar (Landrum et al., 2016), Polyphen (Adzhubei, Jordan, & Sunyaev, 2013), SIFT (Kumar, Henikoff, & Ng, 2009) and MutationTaster (Schwarz, Cooper, Schuelke, & Seelow, 2014). Variant priorization was performed using our own developed variant analysis and priorization software called B-platform (http://www.bitgenia.com/b-platform/) following recent criteria from the *American College of Medical Genetics and Genomics* (ACMG) (Richards et al., 2015) to classify them. Depth of coverage was computed using nonduplicate reads, and samples in which ≥80% of bases were covered at a threshold of ≥8X coverage were considered passing.

For healthy controls we use the ExAC database, which contains 123,136 exome sequences and 15,496 whole genome sequences from unrelated individuals without severe

pediatric disease (gnomad@broadinstitute.org) (Lek et al., 2016), the online archive of Brazilian variants from 609 healthy individuals (http://abraom.ib.usp.br/), and Dr. Marti's private database of over 100 healthy Argentinean controls derived from our recent project (http://apps.bitgenia.com/100exomas).

Confirmation of *GH1* mutation by Sanger sequencing and CAP/CLIA clinical test

We amplified a 4 kb stretch of sequence including the *GH1* locus with the primers: 5'-AAG TGA AAA GCA TCG AGA TGT GT-3' (*GH1* Forward) and 5'-CAG CTA ACT TTT TTG CAT TTT TAG TAC AG-3' (GH1 Reverse). The reaction was run using Phusion-based PCR (New England Biolabs, Ipswich, MA), with an annealing temperature of 67.0°C, and an extension time of 2 minutes. The resulting product was run on a 1% agarose gel, and a band of 4kb was excised and purified using a Qiagen Gel Extraction kit. Five nanograms of the extracted DNA were PCR amplified using primers that span exon 5 of *GH1*: 5'-GGA CAC CTA GTC AGA CAA AAT GAT G-3' (*GH1* Exon 5 Forward) and 5'-TCT CTA CAC CCT GAA GGG GAG-3' (*GH1* Exon 5 Reverse). The products were separated on a 1% agarose gel, and the 300 bp band was excised and purified in the same manner. Sixty ng of DNA at a concentration of 3ng/μL were submitted to the University of Michigan sequencing core for Sanger Sequencing with the following primers:5'-GAC ACC TAG TCA GAC AAA ATG ATG C-3' (*GH1* Sequencing Forward) and 5'-AGG CTG GAA GAT GGC AGC-3' (*GH1* Sequencing Reverse). The chromatograms were analyzed to ensure amplification was specific to *GH1,* avoiding amplification of the paralogous genes *GH2* (*139240)*, CSH1* (*150200)*, CSH2* (*118820) and *CSHL1*(*603515). *GH1* is distinguishable from *GH2* by adenine vs. cytosine at the 589th position of the mRNA. *GH1* is distinguishable from *CSH1* and *CSHL1*, by cytosine vs. guanine at the 658th position of the mRNA. Finally, *GH1* is distinguished from *CSH2* by polymorphic loci starting at position 715. Genomic DNA sequence of *GH1* was based on the GenBank reference sequences NG_011676.1.

Results

Patient characteristics

The clinical features of 51 patients with CPHD or IGHD are summarized in Table 1. The median age of the patients was 9 years (range 1-29 years), and they represent 44 independent pedigrees with no consanguinity. The majority of these patients were diagnosed with CPHD (84 %) and were sporadic cases. There were 3 familial cases including a three-generation Caucasian pedigree with IGHD. Twenty five percent of the cases were native Argentineans or Amerindian descent.

smMIP Sequencing panel

We developed a refined version of the single-molecule molecular inversion probe (smMIPS) capture assay (Hiatt et al., 2013). The panel was designed to cover all coding exons and intron-exon boundaries of 67 selected genes associated with CPHD, IGHD, SOD, and HPE in humans and/or mice (File S1). This panel targets 693 coding exons totaling 174.1 kb of coding sequence. smMIPS capture, library preparation and sequencing was performed for all 120 samples, using specific barcodes for each sample.

To assess smMIPS accuracy, we included DNA from GM12878, a gold-standard reference cell line, with publically available variant calls from deep whole-genome sequencing (WGS) (Zook et al., 2014). For this individual, we obtained 2.1 million read pairs, resulting in median coverage of 154X, and 97.6% of targeted bases reaching ≥8X read depth coverage, and 95.1% of bases reaching ≥40X. Within regions with sufficient coverage, variant calling was highly accurate, with 99.54% SNP/indel variant sensitivity, with an overall genotype concordance of >99.6% (positions with ≥ 8 reads). After instituting genomic DNA quality control for concentration and absorbance ratio (260/280), and, as needed, re-purification, 97% of samples sequenced successfully (defined as 98% of targeted bases at covered by ≥8 reads which is sufficient for sensitivity and specificity in the cell line). On average, 98% of regions of interest were covered >100x. Nine exons were not covered or had an average coverage lower than 10 (Figure 1S).

Identification of *GH1* mutation

We found a *GH1* mutation, in a three-generation pedigree with autosomal dominant growth insufficiency using smMIPS (Figure 2). MRI showed mild anterior pituitary hypoplasia in two patients and a thin pituitary stalk in one of them. We also found the same mutation in an apparently unrelated female patient with IGHD and in her apparently unaffected father who is deceased and no additional details are available. We confirmed proper segregation of the variant in the three generation pedigree with Sanger sequencing. While this was in progress, a new baby was born in the family (III-4). We arranged for a CAP/CLIA clinical test to be conducted so that results could be returned to the physicians. This test revealed that the baby was affected, and GH treatment began immediately. This example provides proof of the principle that the smMIPS can detect clinical relevant mutations in known genes. Patients III-1 and III-3 responded to GH treatment commencing at nine years of age (0.21 mg/kg/w) and four years of age (0.17 mg/kg/w) respectably.

This mutation, C>T c.626G>A p.R209H based on ENST00000323322, has been described previously as p.R183H in several pedigrees and shown to interfere with the secretion

of GH (Deladoey, Stocker, & Mullis, 2001; Gertner, Wajnrajch, & Leibel, 1998; Marino et al., 2003b; Miyata et al., 1997). The numbering in the previous publication was based on assigning the first amino acid of the GH protein following cleavage of the signal peptide.

The frequency of the *PROP1* mutation varies widely by population group, and the rate was previously unknown for Argentina. We found no cases of *PROP1* mutations in this first cohort analyzed by smMIP selection and high throughput sequencing.

Discussion

We developed a targeted next-generation sequencing panel using single molecule molecular inversion probes (smMIPS) to identify mutations in pituitary hormone deficiency patients. smMIPS is a rapid, scalable and economical method for sequencing candidate loci for mutation discovery. smMIPS enables multiplexed sequencing of targets ranging from small gene panels (Hor et al., 2015) to whole exomes (Turner, Lee, Ng, Nickerson, & Shendure, 2009) across very large cohorts for which whole-genome or whole-exome sequencing would be cost-prohibitive. smMIPS have been previously used to screen for *de novo* mutations in autism risk genes, allowing interrogation of much larger cohorts than presently feasible with whole-genome or exome sequencing (Neale et al., 2012; Stessman et al., 2017; Wang et al., 2016). smMIPS sequencing has also recently been applied clinically to test for mutations in the tumor suppressor genes *BRCA1* and *BRCA2* (Neveling et al., 2017) and has demonstrated superior accuracy and turnaround time relative to previous lab-developed testing. We are not aware of systematic screening for pathogenic variants that cause CPHD or IGHD with panels of known genes in Argentina or any other population group.

Isolated growth hormone deficiency is most frequently caused by mutations in the *GH1* gene, especially gene deletions and conversion events stimulated by the array of GH related genes (Mullis, 2011). Pathogenic mutations in the growth hormone releasing hormone receptor, *GHRHR* (Salvatori et al., 2001; Salvatori et al., 1999; Wajnrajch, Gertner, Harbison, Chua, & Leibel, 1996) and *GHSR* have also been reported to cause IGHD (Inoue et al., 2011; Pantel et al., 2009; Pugliese-Pires et al., 2011). IGHD1A and IGHD1B exhibit autosomal recessive mutations in *GH1*, while IGHD2 is characterized by autosomal dominant mutations in *GH1* (Phillips & Cogan, 1994). Individuals with IGHD2 present with variable height deficits and variable pituitary size, and other hormone deficits may emerge. The majority of these dominant cases are caused by mutations in the intron 3 splice donor site, which cause skipping of exon 3 and generation of a 17.5 kDa GH instead of the bioactive 22 or 20 kDa forms (Mullis et al., 2005). The 17.5 kDa form of GH has a dominant negative effect on GH secretion and causes

cell death, explaining the progressive hormone deficiency (McGuinness et al., 2003; Ryther et al., 2003; Shariat, Holladay, Cleary, Phillips, & Patton, 2008). Mutations in exonic splice enhancers also cause increased production of the 17.5 kDa GH. There are a few missense mutations that cause IGHD2, and some of them are likely pathogenic because they affect splicing (Babu et al., 2014).

Our screening uncovered a recurrent *GH1* missense mutation, p.R209H, in a family with IGHD2 and in an unrelated sporadic case of IGHD. This recurrent mutation has been reported in ethnically diverse families with IGHD2 and some sporadic IGHD cases (previously referred to as p.R183H). It was reported in a three generation Turkish pedigree of Kurd ancestry (Deladoey et al., 2001), in two, large, unrelated families of Christian-Arab and Ashkenazi Jewish descent (Hess et al., 2007), and in two unrelated IGHD patients from Argentina (Marino et al., 2003a). Individuals with this variant exhibit a variable phenotype, with carriers of the same family exhibiting height (SDS) ranging from -4.5 to -1.0 (Hess et al., 2007). While all the variant carriers in the familial case reported here had severe short stature, the sporadic case had an apparently unaffected father, consistent with reports of variable expressivity of this allele. No additional pituitary hormone deficiency was found in our patients, and no progression has been reported for other patients with the same variant. All patients responded well to growth hormone replacement therapy.

The exact mechanism whereby the p.R209H GH impairs growth is not clear. However, elegant transfection studies demonstrated that the variant GH protein can be secreted effectively in response to cAMP stimulation, but if co-expressed with the normal protein, secretion is greatly reduced (Deladoey et al., 2001). This suggests that the missense mutation interferes with the aggregation of GH proteins that is necessary to form secretory granules.

The frequency of *PROP1* mutations varies greatly based on ethnicity, with high levels reported in Lithuanian (65%) and Russian (46%) cohorts and less than 1% in patients from the United Kingdom, Germany, Japan and Korea (De Rienzo et al., 2015; Dusatkova et al., 2016; Navardauskaite et al., 2014). The Argentinean population is a mixture of European (67%), Native American (28%), West African (3.6%) and East Asian (1.4%) ancestry, and the European component is predominantly from Spain and Italy (Homburger et al., 2015). The rate of *PROP1* mutations in Argentina was 0/44, which compares well with the low rates of *PROP1* mutations in Spain (Coya et al., 2007) (0/36) and Italy (De Rienzo et al., 2015) (3/126, 2.4%). Slightly higher rates were reported for Portugal (9/36, 25%) (Lemos et al., 2006)) and Brazil (Vieira et al., 2007) (5/29, 17%).

In summary, we developed a gene panel based on single molecule molecular inversion probe sequencing and captured the coding exons of 67 candidate genes in 51 patients with hypopituitarism. We found a mutation in the *GH1* gene that is responsible for familial isolated growth hormone deficiency type II. Identifying these potential variants will make it feasible to predict clinical outcomes from genetic data, which is necessary for patient diagnosis and prognosis, and for assessing the risk of future affected individuals. We believe that the approach described here is cost and time efficient, and should be apply first in molecular diagnosis, follow by CNV assays and whole genome sequencing to provide much needed diagnoses for patients and their families.

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Conflict of Interest Statement

The authors have nothing to disclose. SAC, AZD, AHM, SV, JB, MIPM, AS, MM, IB, DB, AK and JOK

References

Adzhubei, I., Jordan, D. M., & Sunyaev, S. R. (2013). Predicting functional effect of human missense mutations using PolyPhen-2. *Current protocols in human genetics, Chapter 7*, Unit7 20. doi:10.1002/0471142905.hg0720s76

Agarwal, G., Bhatia, V., Cook, S., & Thomas, P. Q. (2000). Adrenocorticotropin deficiency in combined pituitary hormone deficiency patients homozygous for a novel PROP1 deletion. *J Clin Endocrinol Metab, 85*(12), 4556-4561. doi:10.1210/jcem.85.12.7013

Alatzoglou, K. S., & Dattani, M. T. (2010). Genetic causes and treatment of isolated growth hormone deficiency-an update. *Nat Rev Endocrinol, 6*(10), 562-576. doi:10.1038/nrendo.2010.147

Alatzoglou, K. S., Turton, J. P., Kelberman, D., Clayton, P. E., Mehta, A., Buchanan, C., . . . Dattani, M. T. (2009). Expanding the spectrum of mutations in GH1 and GHRHR: genetic screening in a large cohort of patients with congenital isolated growth hormone deficiency. *The Journal of clinical endocrinology and metabolism, 94*(9), 3191-3199. doi:10.1210/jc.2008-2783

Arnhold, I. J., Franca, M. M., Carvalho, L. R., Mendonca, B. B., & Jorge, A. A. (2015). Role of GLI2 in hypopituitarism phenotype. *J Mol Endocrinol, 54*(3), R141-150. doi:10.1530/JME-15-0009

Babu, D., Mellone, S., Fusco, I., Petri, A., Walker, G. E., Bellone, S., . . . Giordano, M. (2014). Novel mutations in the GH gene (GH1) uncover putative splicing regulatory elements. *Endocrinology, 155*(5), 1786-1792. doi:10.1210/en.2013-2146

Ballerini, M. G., Chiesa, A., Scaglia, P., Gruneiro-Papendieck, L., Heinrich, J. J., & Ropelato, M. G. (2010). 17alpha-hydroxyprogesterone and cortisol serum levels in neonates and young children: influence of age, gestational age, gender and methodological procedures. *Journal of pediatric endocrinology & metabolism : JPEM, 23*(1-2), 121-132.

Blum, W. F., Deal, C., Zimmermann, A. G., Shavrikova, E. P., Child, C. J., Quigley, C. A., . . . Rosenfeld, R. G. (2014). Development of additional pituitary hormone deficiencies in pediatric patients originally diagnosed with idiopathic isolated GH deficiency. *Eur J Endocrinol, 170*(1), 13-21. doi:10.1530/EJE-13-0643

EJE-13-0643 [pii]

Bottner, A., Keller, E., Kratzsch, J., Stobbe, H., Weigel, J. F., Keller, A., . . . Pfaffle, R. W. (2004). PROP1 mutations cause progressive deterioration of anterior pituitary function including adrenal insufficiency: a longitudinal analysis. *J Clin Endocrinol Metab, 89*(10), 5256-5265. doi:10.1210/jc.2004-0661

Braslavsky, D., Grinspon, R. P., Ballerini, M. G., Bedecarras, P., Loreti, N., Bastida, G., . . . Bergada, I. (2015). Hypogonadotropic Hypogonadism in Infants with Congenital Hypopituitarism: A Challenge to Diagnose at an Early Stage. *Hormone research in paediatrics, 84*(5), 289-297. doi:10.1159/000439051

Cingolani, P., Patel, V. M., Coon, M., Nguyen, T., Land, S. J., Ruden, D. M., & Lu, X. (2012). Using Drosophila melanogaster as a Model for Genotoxic Chemical Mutational Studies with a New Program, SnpSift. *Frontiers in genetics, 3*, 35. doi:10.3389/fgene.2012.00035

Cogan, J. D., Wu, W., Phillips, J. A., 3rd, Arnhold, I. J., Agapito, A., Fofanova, O. V., . . . Mendonca, B. B. (1998). The PROP1 2-base pair deletion is a common cause of combined pituitary hormone deficiency. *J Clin Endocrinol Metab, 83*(9), 3346-3349. doi:10.1210/jcem.83.9.5142

Coya, R., Vela, A., Perez de Nanclares, G., Rica, I., Castano, L., Busturia, M. A., . . . group, G. (2007). Panhypopituitarism: genetic versus acquired etiological factors. *J Pediatr Endocrinol Metab, 20*(1), 27-36.

Dasen, J. S., Martinez Barbera, J. P., Herman, T. S., Connell, S. O., Olson, L., Ju, B., . . . Rosenfeld, M. G. (2001). Temporal regulation of a paired-like homeodomain repressor/TLE corepressor complex and a related activator is required for pituitary organogenesis. *Genes Dev, 15*(23), 3193-3207. doi:10.1101/gad.932601

Dateki, S., Fukami, M., Sato, N., Muroya, K., Adachi, M., & Ogata, T. (2008). OTX2 mutation in a patient with anophthalmia, short stature, and partial growth hormone deficiency: functional studies using the IRBP, HESX1, and POU1F1 promoters. *J Clin Endocrinol Metab, 93*(10), 3697-3702.

Dateki, S., Fukami, M., Uematsu, A., Kaji, M., Iso, M., Ono, M., . . . Ogata, T. (2010). Mutation and gene copy number analyses of six pituitary transcription factor genes in 71 patients with combined pituitary hormone deficiency: identification of a single patient with LHX4 deletion. *J Clin Endocrinol Metab, 95*(8), 4043-4047. doi:10.1210/jc.2010-0150

Dattani, M. T., Martinez-Barbera, J. P., Thomas, P. Q., Brickman, J. M., Gupta, R., Martensson, I. L., . . . Robinson, I. C. (1998). Mutations in the homeobox gene HESX1/Hesx1 associated with septo-optic dysplasia in human and mouse. *Nature genetics, 19*(2), 125-133. doi:10.1038/477

de Graaff, L. C., Argente, J., Veenma, D. C., Drent, M. L., Uitterlinden, A. G., & Hokken-Koelega, A. C. (2010). PROP1, HESX1, POU1F1, LHX3 and LHX4 mutation and deletion screening and GH1 P89L and IVS3+1/+2 mutation screening in a Dutch nationwide cohort of

patients with combined pituitary hormone deficiency. *Horm Res Paediatr, 73*(5), 363-371. doi:10.1159/000308169

De Rienzo, F., Mellone, S., Bellone, S., Babu, D., Fusco, I., Prodam, F., . . . Italian Study Group on Genetics of, C. (2015). Frequency of genetic defects in combined pituitary hormone deficiency: a systematic review and analysis of a multicentre Italian cohort. *Clin Endocrinol (Oxf), 83*(6), 849-860. doi:10.1111/cen.12849

Deladoey, J., Fluck, C., Buyukgebiz, A., Kuhlmann, B. V., Eble, A., Hindmarsh, P. C., . . . Mullis, P. E. (1999). "Hot spot" in the PROP1 gene responsible for combined pituitary hormone deficiency. *J Clin Endocrinol Metab, 84*(5), 1645-1650. doi:10.1210/jcem.84.5.5681

Deladoey, J., Stocker, P., & Mullis, P. E. (2001). Autosomal dominant GH deficiency due to an Arg183His GH-1 gene mutation: clinical and molecular evidence of impaired regulated GH secretion. *J Clin Endocrinol Metab, 86*(8), 3941-3947. doi:10.1210/jcem.86.8.7723

DePristo, M. A., Banks, E., Poplin, R., Garimella, K. V., Maguire, J. R., Hartl, C., . . . Daly, M. J. (2011). A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nat Genet, 43*(5), 491-498. doi:10.1038/ng.806

ng.806 [pii]

Diaczok, D., Romero, C., Zunich, J., Marshall, I., & Radovick, S. (2008). A Novel Dominant Negative Mutation of OTX2 Associated with Combined Pituitary Hormone Deficiency. *J Clin Endocrinol Metab*.

Dusatkova, P., Pfaffle, R., Brown, M. R., Akulevich, N., Arnhold, I. J., Kalina, M. A., . . . Lebl, J. (2016). Genesis of two most prevalent PROP1 gene variants causing combined pituitary hormone deficiency in 21 populations. *Eur J Hum Genet, 24*(3), 415-420. doi:10.1038/ejhg.2015.126

Fang, Q., George, A. S., Brinkmeier, M. L., Mortensen, A. H., Gergics, P., Cheung, L. Y., . . . Camper, S. A. (2016). Genetics of Combined Pituitary Hormone Deficiency: Roadmap into the Genome Era. *Endocr Rev, 37*(6), 636-675. doi:10.1210/er.2016-1101

Flemming, G. M., Klammt, J., Ambler, G., Bao, Y., Blum, W. F., Cowell, C., . . . Pfaffle, R. W. (2013). Functional characterization of a heterozygous GLI2 missense mutation in

patients with multiple pituitary hormone deficiency. *J Clin Endocrinol Metab, 98*(3), E567-575. doi:10.1210/jc.2012-3224

Fluck, C., Deladoey, J., Rutishauser, K., Eble, A., Marti, U., Wu, W., & Mullis, P. E. (1998). Phenotypic variability in familial combined pituitary hormone deficiency caused by a PROP1 gene mutation resulting in the substitution of Arg-->Cys at codon 120 (R120C). *J Clin Endocrinol Metab, 83*(10), 3727-3734. doi:10.1210/jcem.83.10.5172

Fofanova, O., Takamura, N., Kinoshita, E., Vorontsov, A., Vladimirova, V., Dedov, I., . . . Yamashita, S. (2000). MR imaging of the pituitary gland in children and young adults with congenital combined pituitary hormone deficiency associated with PROP1 mutations. *AJR Am J Roentgenol, 174*(2), 555-559. doi:10.2214/ajr.174.2.1740555

Fofanova, O. V., Takamura, N., Kinoshita, E., Yoshimoto, M., Tsuji, Y., Peterkova, V. A., . . . Yamashita, S. (1998). Rarity of PIT1 involvement in children from Russia with combined pituitary hormone deficiency. *Am J Med Genet, 77*(5), 360-365.

Franca, M. M., Jorge, A. A., Carvalho, L. R., Costalonga, E. F., Vasques, G. A., Leite, C. C., . . . Arnhold, I. J. (2010). Novel heterozygous nonsense GLI2 mutations in patients with hypopituitarism and ectopic posterior pituitary lobe without holoprosencephaly. *J Clin Endocrinol Metab, 95*(11), E384-391. doi:10.1210/jc.2010-1050

Gage, P. J., Brinkmeier, M. L., Scarlett, L. M., Knapp, L. T., Camper, S. A., & Mahon, K. A. (1996). The Ames dwarf gene, df, is required early in pituitary ontogeny for the extinction of Rpx transcription and initiation of lineage-specific cell proliferation. *Mol Endocrinol, 10*(12), 1570-1581.

Gertner, J. M., Wajnrajch, M. P., & Leibel, R. L. (1998). Genetic defects in the control of growth hormone secretion. *Horm Res, 49 Suppl 1*, 9-14.

Halasz, Z., Toke, J., Patocs, A., Bertalan, R., Tombol, Z., Sallai, A., . . . Racz, K. (2006). High prevalence of PROP1 gene mutations in Hungarian patients with childhood-onset combined anterior pituitary hormone deficiency. *Endocrine, 30*(3), 255-260. doi:10.1007/s12020-006-0002-7

Hess, O., Hujeirat, Y., Wajnrajch, M. P., Allon-Shalev, S., Zadik, Z., Lavi, I., & Tenenbaum-Rakover, Y. (2007). Variable phenotypes in familial isolated growth hormone deficiency

caused by a G6664A mutation in the GH-1 gene. *The Journal of clinical endocrinology and metabolism, 92*(11), 4387-4393. doi:10.1210/jc.2007-0684

Hiatt, J. B., Pritchard, C. C., Salipante, S. J., O'Roak, B. J., & Shendure, J. (2013). Single molecule molecular inversion probes for targeted, high-accuracy detection of low-frequency variation. *Genome Res, 23*(5), 843-854. doi:10.1101/gr.147686.112

Homburger, J. R., Moreno-Estrada, A., Gignoux, C. R., Nelson, D., Sanchez, E., Ortiz-Tello, P., . . . Bustamante, C. D. (2015). Genomic Insights into the Ancestry and Demographic History of South America. *PLoS Genet, 11*(12), e1005602. doi:10.1371/journal.pgen.1005602

Hor, H., Francescatto, L., Bartesaghi, L., Ortega-Cubero, S., Kousi, M., Lorenzo-Betancor, O., . . . Estivill, X. (2015). Missense mutations in TENM4, a regulator of axon guidance and central myelination, cause essential tremor. *Hum Mol Genet, 24*(20), 5677-5686. doi:10.1093/hmg/ddv281

Inoue, H., Kangawa, N., Kinouchi, A., Sakamoto, Y., Kimura, C., Horikawa, R., . . . Japan Growth Genome, C. (2011). Identification and functional analysis of novel human growth hormone secretagogue receptor (GHSR) gene mutations in Japanese subjects with short stature. *J Clin Endocrinol Metab, 96*(2), E373-378. doi:10.1210/jc.2010-1570

Kandemir, N., Vuralli, D., Taskiran, E., Gonc, N., Ozon, A., Alikasifoglu, A., & Yilmaz, E. (2012). Frequency of mutations in PROP-1 gene in Turkish children with combined pituitary hormone deficiency. *Turk J Pediatr, 54*(6), 570-575.

Karczewski, K. J., Weisburd, B., Thomas, B., Solomonson, M., Ruderfer, D. M., Kavanagh, D., . . . MacArthur, D. G. (2017). The ExAC browser: displaying reference data information from over 60 000 exomes. *Nucleic acids research, 45*(D1), D840-D845. doi:10.1093/nar/gkw971

Kelberman, D., Rizzoti, K., Avilion, A., Bitner-Glindzicz, M., Cianfarani, S., Collins, J., . . . Dattani, M. T. (2006). Mutations within Sox2/SOX2 are associated with abnormalities in the hypothalamo-pituitary-gonadal axis in mice and humans. *The Journal of clinical investigation, 116*(9), 2442-2455. doi:10.1172/JCI28658

Kim, S. S., Kim, Y., Shin, Y. L., Kim, G. H., Kim, T. U., & Yoo, H. W. (2003). Clinical characteristics and molecular analysis of PIT1, PROP1,LHX3, and HESX1 in combined pituitary hormone

546 deficiency patients with abnormal pituitary MR imaging. *Horm Res, 60*(6), 277-283.
547 doi:74245
548 Kumar, P., Henikoff, S., & Ng, P. C. (2009). Predicting the effects of coding non-synonymous
549 variants on protein function using the SIFT algorithm. *Nat Protoc, 4*(7), 1073-1081.
550 doi:10.1038/nprot.2009.86
551 nprot.2009.86 [pii]
552 Landrum, M. J., Lee, J. M., Benson, M., Brown, G., Chao, C., Chitipiralla, S., . . . Maglott, D. R.
553 (2016). ClinVar: public archive of interpretations of clinically relevant variants. *Nucleic*
554 *acids research, 44*(D1), D862-868. doi:10.1093/nar/gkv1222
555 Lebl, J., Vosahlo, J., Pfaeffle, R. W., Stobbe, H., Cerna, J., Novotna, D., . . . Blum, W. F. (2005).
556 Auxological and endocrine phenotype in a population-based cohort of patients with
557 PROP1 gene defects. *Eur J Endocrinol, 153*(3), 389-396. doi:10.1530/eje.1.01989
558 Lek, M., Karczewski, K. J., Minikel, E. V., Samocha, K. E., Banks, E., Fennell, T., . . . Exome
559 Aggregation, C. (2016). Analysis of protein-coding genetic variation in 60,706 humans.
560 *Nature, 536*(7616), 285-291. doi:10.1038/nature19057
561 Lemos, M. C., Gomes, L., Bastos, M., Leite, V., Limbert, E., Carvalho, D., . . . Carvalheiro, M.
562 (2006). PROP1 gene analysis in Portuguese patients with combined pituitary hormone
563 deficiency. *Clin Endocrinol (Oxf), 65*(4), 479-485. doi:10.1111/j.1365-2265.2006.02617.x
564 Li, H. (2013). Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. .
565 *arXiv*.
566 Marino, R., Chaler, E., Warman, M., Ciaccio, M., Berensztein, E., Rivarola, M. A., & Belgorosky,
567 A. (2003a). The serum growth hormone (GH) response to provocative tests is dependent
568 on type of assay in autosomal dominant isolated GH deficiency because of an
569 ARG(183)HIS (R183H) GH-I gene mutation. *Clinical chemistry, 49*(6 Pt 1), 1002-1005.
570 Marino, R., Chaler, E., Warman, M., Ciaccio, M., Berensztein, E., Rivarola, M. A., & Belgorosky,
571 A. (2003b). The serum growth hormone (GH) response to provocative tests is dependent
572 on type of assay in autosomal dominant isolated GH deficiency because of an
573 ARG(183)HIS (R183H) GH-I gene mutation. *Clin Chem, 49*(6 Pt 1), 1002-1005.

574 Matsuo, I., Kuratani, S., Kimura, C., Takeda, N., & Aizawa, S. (1995). Mouse Otx2 functions in the
575 formation and patterning of rostral head. *Genes Dev, 9*(21), 2646-2658.
576 McGuinness, L., Magoulas, C., Sesay, A. K., Mathers, K., Carmignac, D., Manneville, J. B., . . .
577 Robinson, I. C. (2003). Autosomal dominant growth hormone deficiency disrupts
578 secretory vesicles in vitro and in vivo in transgenic mice. *Endocrinology, 144*(2), 720-731.
579 doi:10.1210/en.2002-220847
580 McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., . . . DePristo, M.
581 A. (2010). The Genome Analysis Toolkit: a MapReduce framework for analyzing next-
582 generation DNA sequencing data. *Genome Res, 20*(9), 1297-1303.
583 doi:10.1101/gr.107524.110
584 gr.107524.110 [pii]
585 McLennan, K., Jeske, Y., Cotterill, A., Cowley, D., Penfold, J., Jones, T., . . . Choong, C. (2003).
586 Combined pituitary hormone deficiency in Australian children: clinical and genetic
587 correlates. *Clin Endocrinol (Oxf), 58*(6), 785-794.
588 Mehta, A., & Dattani, M. T. (2008). Developmental disorders of the hypothalamus and pituitary
589 gland associated with congenital hypopituitarism. *Best Pract Res Clin Endocrinol Metab,*
590 *22*(1), 191-206.
591 Mendonca, B. B., Osorio, M. G., Latronico, A. C., Estefan, V., Lo, L. S., & Arnhold, I. J. (1999).
592 Longitudinal hormonal and pituitary imaging changes in two females with combined
593 pituitary hormone deficiency due to deletion of A301,G302 in the PROP1 gene. *J Clin*
594 *Endocrinol Metab, 84*(3), 942-945. doi:10.1210/jcem.84.3.5537
595 Miyata, I., Cogan, J. D., Prince, M. A., Kamijo, T., Ogawa, M., & Phillips, J. A., 3rd. (1997).
596 Detection of growth hormone gene defects by dideoxy fingerprinting (ddF). *Endocr J,*
597 *44*(1), 149-154.
598 Mortensen, A. H., MacDonald, J. W., Ghosh, D., & Camper, S. A. (2011). Candidate genes for
599 panhypopituitarism identified by gene expression profiling. *Physiol Genomics, 43*(19),
600 1105-1116. doi:10.1152/physiolgenomics.00080.2011

Mortensen, A. H., Schade, V., Lamonerie, T., & Camper, S. A. (2015). Deletion of OTX2 in neural ectoderm delays anterior pituitary development. *Hum Mol Genet, 24*(4), 939-953. doi:10.1093/hmg/ddu506

Mullis, P. E. (2011). Genetics of GHRH, GHRH-receptor, GH and GH-receptor: its impact on pharmacogenetics. *Best Pract Res Clin Endocrinol Metab, 25*(1), 25-41. doi:10.1016/j.beem.2010.06.006

Mullis, P. E., Robinson, I. C., Salemi, S., Eble, A., Besson, A., Vuissoz, J. M., . . . Binder, G. (2005). Isolated autosomal dominant growth hormone deficiency: an evolving pituitary deficit? A multicenter follow-up study. *J Clin Endocrinol Metab, 90*(4), 2089-2096. doi:10.1210/jc.2004-1280

Navardauskaite, R., Dusatkova, P., Obermannova, B., Pfaeffle, R. W., Blum, W. F., Adukauskiene, D., . . . Lebl, J. (2014). High prevalence of PROP1 defects in Lithuania: phenotypic findings in an ethnically homogenous cohort of patients with multiple pituitary hormone deficiency. *J Clin Endocrinol Metab, 99*(1), 299-306. doi:10.1210/jc.2013-3090

Neale, B. M., Kou, Y., Liu, L., Ma'ayan, A., Samocha, K. E., Sabo, A., . . . Daly, M. J. (2012). Patterns and rates of exonic de novo mutations in autism spectrum disorders. *Nature, 485*(7397), 242-245. doi:10.1038/nature11011

Neveling, K., Mensenkamp, A. R., Derks, R., Kwint, M., Ouchene, H., Steehouwer, M., . . . Hoischen, A. (2017). BRCA Testing by Single-Molecule Molecular Inversion Probes. *Clin Chem, 63*(2), 503-512. doi:10.1373/clinchem.2016.263897

Nishida, A., Furukawa, A., Koike, C., Tano, Y., Aizawa, S., Matsuo, I., & Furukawa, T. (2003). Otx2 homeobox gene controls retinal photoreceptor cell fate and pineal gland development. *Nat Neurosci, 6*(12), 1255-1263.

Otto, A. P., Franca, M. M., Correa, F. A., Costalonga, E. F., Leite, C. C., Mendonca, B. B., . . . Jorge, A. A. (2015). Frequent development of combined pituitary hormone deficiency in patients initially diagnosed as isolated growth hormone deficiency: a long term follow-up of patients from a single center. *Pituitary, 18*(4), 561-567. doi:10.1007/s11102-014-0610-9

Pantel, J., Legendre, M., Nivot, S., Morisset, S., Vie-Luton, M. P., le Bouc, Y., . . . Amselem, S. (2009). Recessive isolated growth hormone deficiency and mutations in the ghrelin receptor. *J Clin Endocrinol Metab, 94*(11), 4334-4341. doi:10.1210/jc.2009-1327

Perez Millan, M. I., Brinkmeier, M. L., Mortensen, A. H., & Camper, S. A. (2016). PROP1 triggers epithelial-mesenchymal transition-like process in pituitary stem cells. *Elife, 5*. doi:10.7554/eLife.14470

Pernasetti, F., Toledo, S. P., Vasilyev, V. V., Hayashida, C. Y., Cogan, J. D., Ferrari, C., . . . Mellon, P. L. (2000). Impaired adrenocorticotropin-adrenal axis in combined pituitary hormone deficiency caused by a two-base pair deletion (301-302delAG) in the prophet of Pit-1 gene. *J Clin Endocrinol Metab, 85*(1), 390-397. doi:10.1210/jcem.85.1.6324

Pfaeffle, R. W., Savage, J. J., Hunter, C. S., Palme, C., Ahlmann, M., Kumar, P., . . . Rhodes, S. J. (2007). Four novel mutations of the LHX3 gene cause combined pituitary hormone deficiencies with or without limited neck rotation. *J Clin Endocrinol Metab, 92*(5), 1909-1919. doi:10.1210/jc.2006-2177

Phillips, J. A., 3rd, & Cogan, J. D. (1994). Genetic basis of endocrine disease. 6. Molecular basis of familial human growth hormone deficiency. *J Clin Endocrinol Metab, 78*(1), 11-16. doi:10.1210/jcem.78.1.8288694

Pugliese-Pires, P. N., Fortin, J. P., Arthur, T., Latronico, A. C., Mendonca, B. B., Villares, S. M., . . . Jorge, A. A. (2011). Novel inactivating mutations in the GH secretagogue receptor gene in patients with constitutional delay of growth and puberty. *Eur J Endocrinol, 165*(2), 233-241. doi:10.1530/EJE-11-0168

Radovick, S., Nations, M., Du, Y., Berg, L. A., Weintraub, B. D., & Wondisford, F. E. (1992). A mutation in the POU-homeodomain of Pit-1 responsible for combined pituitary hormone deficiency. *Science, 257*(5073), 1115-1118.

Rainbow, L. A., Rees, S. A., Shaikh, M. G., Shaw, N. J., Cole, T., Barrett, T. G., & Kirk, J. M. (2005). Mutation analysis of POUF-1, PROP-1 and HESX-1 show low frequency of mutations in children with sporadic forms of combined pituitary hormone deficiency and septo-optic dysplasia. *Clin Endocrinol (Oxf), 62*(2), 163-168. doi:10.1111/j.1365-2265.2004.02189.x

Raivio, T., Avbelj, M., McCabe, M. J., Romero, C. J., Dwyer, A. A., Tommiska, J., . . . Pitteloud, N. (2012). Genetic overlap in Kallmann syndrome, combined pituitary hormone deficiency, and septo-optic dysplasia. *J Clin Endocrinol Metab, 97*(4), E694-699. doi:10.1210/jc.2011-2938

Reynaud, R., Gueydan, M., Saveanu, A., Vallette-Kasic, S., Enjalbert, A., Brue, T., & Barlier, A. (2006). Genetic screening of combined pituitary hormone deficiency: experience in 195 patients. *J Clin Endocrinol Metab, 91*(9), 3329-3336. doi:10.1210/jc.2005-2173

Richards, S., Aziz, N., Bale, S., Bick, D., Das, S., Gastier-Foster, J., . . . Rehm, H. L. (2015). Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genetics in medicine : official journal of the American College of Medical Genetics, 17*(5), 405-424. doi:10.1038/gim.2015.30

Riepe, F. G., Partsch, C. J., Blankenstein, O., Monig, H., Pfaffle, R. W., & Sippell, W. G. (2001). Longitudinal imaging reveals pituitary enlargement preceding hypoplasia in two brothers with combined pituitary hormone deficiency attributable to PROP1 mutation. *J Clin Endocrinol Metab, 86*(9), 4353-4357. doi:10.1210/jcem.86.9.7828

Rosenbloom, A. L., Almonte, A. S., Brown, M. R., Fisher, D. A., Baumbach, L., & Parks, J. S. (1999). Clinical and biochemical phenotype of familial anterior hypopituitarism from mutation of the PROP1 gene. *J Clin Endocrinol Metab, 84*(1), 50-57. doi:10.1210/jcem.84.1.5366

Ryther, R. C., McGuinness, L. M., Phillips, J. A., 3rd, Moseley, C. T., Magoulas, C. B., Robinson, I. C., & Patton, J. G. (2003). Disruption of exon definition produces a dominant-negative growth hormone isoform that causes somatotroph death and IGHD II. *Hum Genet, 113*(2), 140-148. doi:10.1007/s00439-003-0949-x

Salvatori, R., Fan, X., Phillips, J. A., 3rd, Espigares-Martin, R., Martin De Lara, I., Freeman, K. L., . . . Levine, M. A. (2001). Three new mutations in the gene for the growth hormone (gh)-releasing hormone receptor in familial isolated gh deficiency type ib. *J Clin Endocrinol Metab, 86*(1), 273-279. doi:10.1210/jcem.86.1.7156

685 Salvatori, R., Hayashida, C. Y., Aguiar-Oliveira, M. H., Phillips, J. A., 3rd, Souza, A. H., Gondo, R.
686 G., . . . Levine, M. A. (1999). Familial dwarfism due to a novel mutation of the growth
687 hormone-releasing hormone receptor gene. *J Clin Endocrinol Metab, 84*(3), 917-923.
688 doi:10.1210/jcem.84.3.5599
689 Schwarz, J. M., Cooper, D. N., Schuelke, M., & Seelow, D. (2014). MutationTaster2: mutation
690 prediction for the deep-sequencing age. *Nature methods, 11*(4), 361-362.
691 doi:10.1038/nmeth.2890
692 Shariat, N., Holladay, C. D., Cleary, R. K., Phillips, J. A., 3rd, & Patton, J. G. (2008). Isolated
693 growth hormone deficiency type II caused by a point mutation that alters both splice
694 site strength and splicing enhancer function. *Clin Genet, 74*(6), 539-545.
695 doi:10.1111/j.1399-0004.2008.01042.x
696 Sobrier, M. L., Tsai, Y. C., Perez, C., Leheup, B., Bouceba, T., Duquesnoy, P., . . . Amselem, S.
697 (2016). Functional characterization of a human POU1F1 mutation associated with
698 isolated growth hormone deficiency: a novel etiology for IGHD. *Hum Mol Genet, 25*(3),
699 472-483. doi:10.1093/hmg/ddv486
700 Sornson, M. W., Wu, W., Dasen, J. S., Flynn, S. E., Norman, D. J., O'Connell, S. M., . . . Rosenfeld,
701 M. G. (1996). Pituitary lineage determination by the Prophet of Pit-1 homeodomain
702 factor defective in Ames dwarfism. *Nature, 384*(6607), 327-333.
703 Stessman, H. A., Xiong, B., Coe, B. P., Wang, T., Hoekzema, K., Fenckova, M., . . . Eichler, E. E.
704 (2017). Targeted sequencing identifies 91 neurodevelopmental-disorder risk genes with
705 autism and developmental-disability biases. *Nat Genet*. doi:10.1038/ng.3792
706 Tajima, T., Ohtake, A., Hoshino, M., Amemiya, S., Sasaki, N., Ishizu, K., & Fujieda, K. (2008).
707 OTX2 loss of function mutation causes anophthalmia and combined pituitary hormone
708 deficiency with a small anterior and ectopic posterior pituitary. *J Clin Endocrinol Metab*.
709 Takagi, M., Ishii, T., Inokuchi, M., Amano, N., Narumi, S., Asakura, Y., . . . Hasegawa, T. (2012).
710 Gradual loss of ACTH due to a novel mutation in LHX4: comprehensive mutation
711 screening in Japanese patients with congenital hypopituitarism. *PLoS One, 7*(9), e46008.
712 doi:10.1371/journal.pone.0046008

713 Tatsumi, K., Miyai, K., Notomi, T., Kaibe, K., Amino, N., Mizuno, Y., & Kohno, H. (1992).
714 Cretinism with combined hormone deficiency caused by a mutation in the PIT1 gene.
715 *Nat Genet, 1*(1), 56-58.
716 Turner, E. H., Lee, C., Ng, S. B., Nickerson, D. A., & Shendure, J. (2009). Massively parallel exon
717 capture and library-free resequencing across 16 genomes. *Nat Methods, 6*(5), 315-316.
718 doi:10.1038/nmeth.f.248
719 Turton, J. P., Mehta, A., Raza, J., Woods, K. S., Tiulpakov, A., Cassar, J., . . . Dattani, M. T. (2005).
720 Mutations within the transcription factor PROP1 are rare in a cohort of patients with
721 sporadic combined pituitary hormone deficiency (CPHD). *Clin Endocrinol (Oxf), 63*(1), 10-
722 18. doi:10.1111/j.1365-2265.2005.02291.x
723 Turton, J. P., Reynaud, R., Mehta, A., Torpiano, J., Saveanu, A., Woods, K. S., . . . Dattani, M. T.
724 (2005). Novel mutations within the POU1F1 gene associated with variable combined
725 pituitary hormone deficiency. *J Clin Endocrinol Metab, 90*(8), 4762-4770.
726 doi:10.1210/jc.2005-0570
727 Turton, J. P., Strom, M., Langham, S., Dattani, M. T., & Le Tissier, P. (2012). Two novel mutations
728 in the POU1F1 gene generate null alleles through different mechanisms leading to
729 combined pituitary hormone deficiency. *Clin Endocrinol (Oxf), 76*(3), 387-393.
730 doi:10.1111/j.1365-2265.2011.04236.x
731 Van der Auwera, G. A., Carneiro, M. O., Hartl, C., Poplin, R., Del Angel, G., Levy-Moonshine, A., .
732 . . DePristo, M. A. (2013). From FastQ data to high confidence variant calls: the Genome
733 Analysis Toolkit best practices pipeline. *Current protocols in bioinformatics, 43*, 11 10 11-
734 33. doi:10.1002/0471250953.bi1110s43
735 Vieira, T. C., Boldarine, V. T., & Abucham, J. (2007). Molecular analysis of PROP1, PIT1, HESX1,
736 LHX3, and LHX4 shows high frequency of PROP1 mutations in patients with familial
737 forms of combined pituitary hormone deficiency. *Arq Bras Endocrinol Metabol, 51*(7),
738 1097-1103.
739 Wajnrajch, M. P., Gertner, J. M., Harbison, M. D., Chua, S. C., Jr., & Leibel, R. L. (1996). Nonsense
740 mutation in the human growth hormone-releasing hormone receptor causes growth
741 failure analogous to the little (lit) mouse. *Nat Genet, 12*(1), 88-90.

Wang, T., Guo, H., Xiong, B., Stessman, H. A., Wu, H., Coe, B. P., . . . Eichler, E. E. (2016). De novo genic mutations among a Chinese autism spectrum disorder cohort. *Nat Commun, 7*, 13316. doi:10.1038/ncomms13316

Wit, J. M., Oostdijk, W., Losekoot, M., van Duyvenvoorde, H. A., Ruivenkamp, C. A., & Kant, S. G. (2016). MECHANISMS IN ENDOCRINOLOGY: Novel genetic causes of short stature. *Eur J Endocrinol, 174*(4), R145-173. doi:10.1530/EJE-15-0937

Wu, W., Cogan, J. D., Pfaffle, R. W., Dasen, J. S., Frisch, H., O'Connell, S. M., . . . Rosenfeld, M. G. (1998). Mutations in PROP1 cause familial combined pituitary hormone deficiency. *Nat Genet, 18*(2), 147-149.

Yoon, J. K., Ahn, J., Kim, H. S., Han, S. M., Jang, H., Lee, M. G., . . . Bang, D. (2015). microDuMIP: target-enrichment technique for microarray-based duplex molecular inversion probes. *Nucleic Acids Res, 43*(5), e28. doi:10.1093/nar/gku1188

Zook, J. M., Chapman, B., Wang, J., Mittelman, D., Hofmann, O., Hide, W., & Salit, M. (2014). Integrating human sequence data sets provides a resource of benchmark SNP and indel genotype calls. *Nat Biotechnol, 32*(3), 246-251. doi:10.1038/nbt.2835

Figure Legends

Figure 1: Bioinformatics pipeline and variant filtering strategy. Each step in the analysis of raw sequencing reads to development of a candidate variant list are indicated.

Figure 2: Pedigree and sequencing chromatograms. (A) Pedigree indicates autosomal dominant inheritance. The index patients are indicated with arrows. (B) Genome viewer detection of heterozygous G1664A (C>T) on reverse complement. (C) A sequence chromatogram showing the *GH1* (c.626G>A; p.R209H) mutation. In the chromatogram, the pathogenic variant is indicated with an arrow.

Tables

Table 1: Characteristics of the study subjects

Table 2: Clinical data of the families evaluated

Supplemental Data

Supplemental File 1: Genes on the array and probe sequences

Figure 1S: **Mean read depth for each gene, each exon targeted in the smMIPS panel**. Box plots of mean read depth of the targeted exons.

Table 1: Characteristics of the study subjects

Total patients	51
Age	9 (1-29)
Median age (range)	10.8
Mean age	28 (55%)
Gender	23 (45%)
Male	13 (25%)
Female	38 (75%)
Ethnicity	8 (16%)
Native	43 (84%)
Caucasian	3 (10 affected)
Diagnosis	41
IGHD	51 (100%)
CPHD	30 (59%)
Cases	31 (61%)
Familial	13 (25%)
Sporadic	9 (18%)
Pituitary Hormone Deficiency	2 (4%)
GH deficiency	13
ACTH deficiency	8
TSH deficiency	3
Gonadotropin deficiency	9
PRL deficiency	3
ADH deficiency	30
MRI: Pituitary stalk	8
Absent	11
Thin	16
Interrupted	9

Normal

MRI: Anterior Pituitary

Absent

Hypoplasia

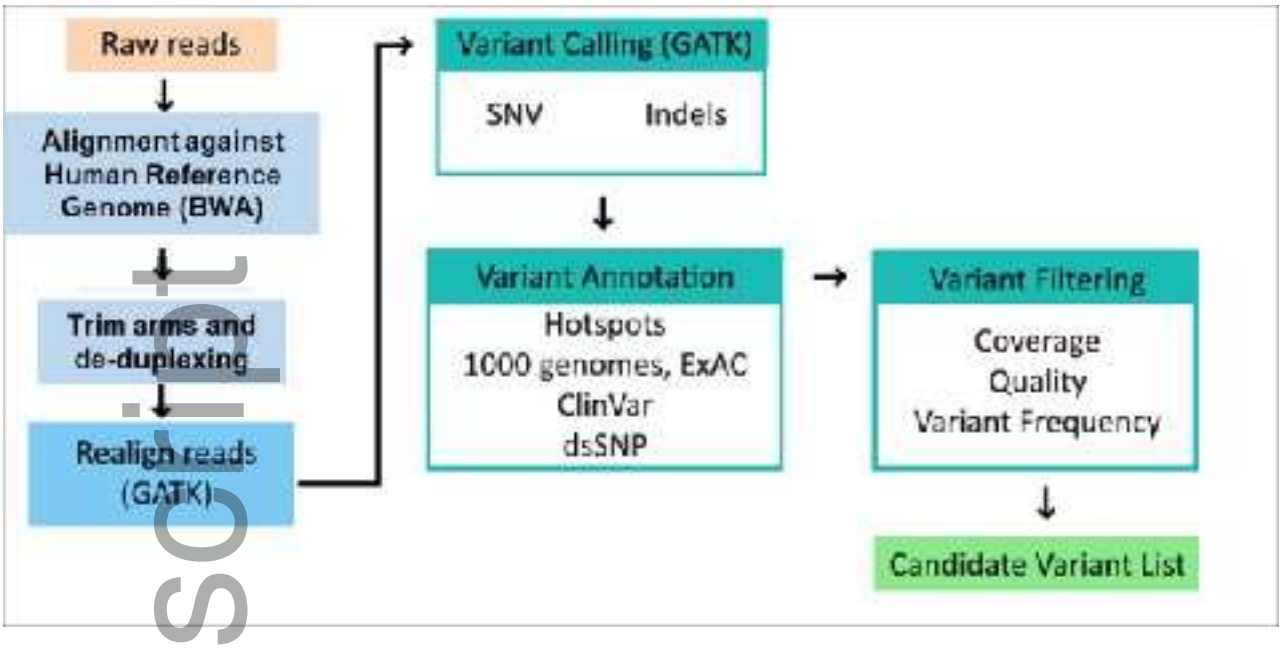
Normal

MRI: Posterior Pituitary

Absent

Ectopic

Normal



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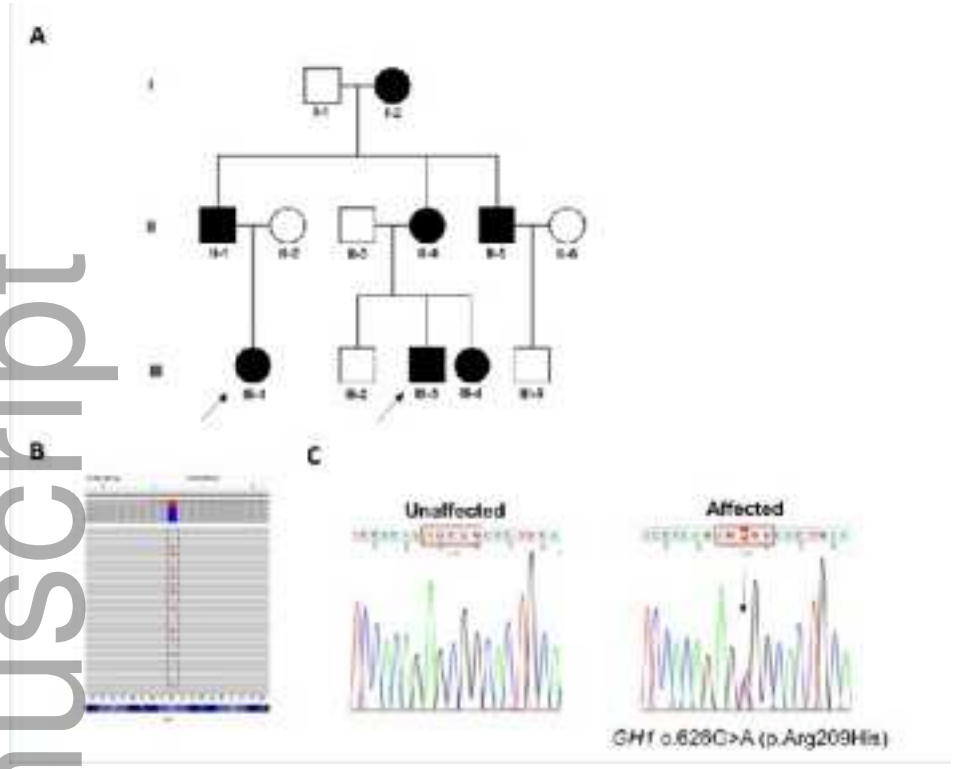

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