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Genetic Evaluation Supports Differential Diagnosis in Adolescent Patients with Delayed Puberty

Tansit Saengkaew[1,2], Heena R Patel[1,3], Kausik Banerjee[4], Gary Butler[5,6], Mehul T Dattani[5,6,7], Michael McGuigan[8], Helen L Storr[1,9], Ruben H Willemsen[9], Leo Dunkel[1,9], Sasha R Howard[1,9]

[1] Centre for Endocrinology, William Harvey Research Institute, Barts and the London School of Medicine and Dentistry, Queen Mary University of London, London, UK

[2] Endocrinology Unit, Department of Paediatrics, Faculty of Medicine, Prince of Songkla University, Songkhla, Thailand

[3] Norwich Medical School, Department of Medicine and Health Sciences, University of East Anglia, Norfolk, UK

[4]Department of Paediatrics, Barking, Havering and Redbridge University Hospitals NHS Trust, London, UK

[5]Department of Paediatric and Adolescent Endocrinology, University College London Hospital NHS Foundation Trust, London, UK

[6] UCL GOS Institute of Child Health, University College London, London, UK

[7] Department of Paediatric Endocrinology, Great Ormond Street Hospital for Children NHS Foundation Trust, London, UK

[8]Department of Paediatrics, Countess of Chester NHS Foundation Trust, Chester, UK

[9]Department of Paediatric Endocrinology, Barts Health NHS Trust, London, UK

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*Corresponding author: Gary Butler, [5]Department of Paediatric and Adolescent Endocrinology, University College London Hospital NHS Foundation Trust, London, UK

[6] UCL GOS Institute of Child Health, University College London, London, UK

E-mail: gary.butler@ucl.ac.uk

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Abstract

Context: Pubertal delay can be the clinical presentation of both idiopathic hypogonadotropic hypogonadism (IHH) and self-limited delayed puberty (SLDP). Distinction between these conditions is a common but important diagnostic challenge in adolescents.

Objective: To assess whether gene panel testing can assist with clinical differential diagnosis, to allow accurate and timely management of delayed puberty patients.

Design: Retrospective study

Methods: Patients presenting with delayed puberty to UK Paediatric services, followed up to final diagnosis, were included. Whole-exome sequencing was analysed using a virtual panel of genes previously reported to cause either IHH or SLDP to identify rare, predicted deleterious variants. Deleterious variants were verified by *in silico* prediction tools. The correlation between clinical and genotype diagnosis was analysed.

Results: Forty-six patients were included, 54% with a final clinical diagnosis of SLDP and 46% with IHH. Red flags signs of IHH were present in only 3 patients. Fifteen predicted deleterious variants in 12 genes were identified in 33% of the cohort, with most inherited in a heterozygous manner. A fair correlation between final clinical diagnosis and genotypic diagnosis was found. Panel testing was able to confirm a diagnosis of IHH in patients with pubertal delay. Genetic analysis identified three patients with IHH that had been previously diagnosed as SLDP.

Conclusion: This study supports the use of targeted exome sequencing in the clinical setting to aid the differential diagnosis between IHH and SLDP in adolescents presenting with pubertal delay. Genetic evaluation thus facilitates earlier and more precise diagnosis, allowing clinicians to direct treatment appropriately.

Introduction

Delayed puberty is a common problem in the paediatric endocrinology clinic, affecting over 2% of adolescents. This condition is diagnosed when children enter puberty 2 to 2.5 SD later than the population average (traditionally, after the age of 14 years in boys and 13 years in girls) (1). Several underlying aetiologies cause pubertal delay, including idiopathic hypogonadotropic hypogonadism (IHH) and hypergonadotropic hypogonadism. However, the most common cause of pubertal delay is self-limited, or constitutional, delayed puberty (SLDP), a functional hypogonadotropic state where individuals enter puberty late, but are post-pubertal by the time they reach adulthood. Hypergonadotropic hypogonadism can be easily excluded by hormonal profiles. The differential diagnosis between SLDP and IHH, however, is often difficult, as both conditions may present with essentially the same clinical and hormonal features (2). Whilst a variety of clinical and biochemical investigations are available to make the diagnosis, none of these can reliably distinguish between those patients who will spontaneously enter and progress in a normal manner through puberty (i.e. SLDP), and those who will require medical induction of puberty and reproductive therapies (i.e. IHH) (3, 4).

This is a vital clinical distinction to make, as if IHH is diagnosed, treatment modalities to allow optimisation of future fertility (particularly for boys) can be used – in the form of gonadotropins rather than sex steroids – for induction of puberty (5), and commenced earlier than the puberty induction regimen used for SLDP patients (6).

Observational studies have shown that SLDP is a familial condition, with the majority of pedigrees displaying autosomal dominant inheritance (with or without complete penetrance) (7, 8). Additionally, 79% of patients have a positive family history of SLDP without any family members with IHH, providing clinicians with further evidence of the diagnosis (7, 9-11). In

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contrast, IHH can be inherited via several modes of inheritance including autosomal dominant, autosomal recessive, X-linked or *de novo* mutation (12, 13). Moreover, in IHH families variable penetrance is commonly seen, probably due in part to oligogenic inheritance of the disease (14, 15). To date, more than 40 genes have been identified that carry mutations which lead or contribute to conditions of IHH (13). Similarly, over the last five years, a smaller but increasing number of genes have been discovered that underlie SLDP by our and other groups through next-generation sequencing (16, 17). Crucially, whilst there is some overlap in the genetic background of these conditions, the majority of mutations are distinct between the two diseases (15). Therefore, genetic analysis using exome sequencing of a panel of known genes could be used to assist a clinician in distinguishing those adolescents with severe gonadotropin deficiency from those with isolated delayed puberty, allowing delivery of accurate and timely treatment to patients. However, this potential utility has yet to be assessed in a clinical cohort of patients presenting in adolescence with delayed puberty of undiagnosed aetiology.

Therefore, in this study, we investigated the burden of genetic variants in a real world, mixed ethnicity cohort of UK adolescent patients presenting with pubertal delay, in order to validate the use of genetic analysis of known causal genes to confirm the diagnosis of IHH or SLDP. We also evaluated the utility of genetic criteria to assist clinicians in confirming the diagnosis of IHH.

Materials and methods

Patients

This study investigated a cohort of patients who were referred for genetic evaluation for delayed puberty from Paediatric Endocrinology and Paediatric services around the UK from 2015-2020

under a NIHR clinical research network portfolio study (Genetic Factors Affecting the Timing of Puberty, CPMS ID 30730).

Delayed puberty was defined as the onset of Tanner stage G2 (testicular volume >3 ml) at >14 years in boys or Tanner stage B2 at >13.0 year in girls (i.e. two SD later than average pubertal development). Chronic illness was excluded by detailed medical history, physical examination, and routine laboratory investigations, and hormonal investigations (basal and stimulated serum LH and FSH, serum testosterone, serum oestradiol, inhibin B) were evaluated to determine the clinical diagnosis. Pubertal progression was also assessed to establish the clinical diagnosis: those who were near to completion or had completed pubertal development (Tanner staging of at least G4 or B4) by 18 years of age and not requiring further sex steroid treatment were diagnosed as SLDP, whereas patients who had not completed puberty by the age of 18 years or had arrested puberty during prior to the age of 18 years were defined as IHH. Patients who did not have a definite diagnosis of SLDP or IHH, as they were still undergoing a period of clinical follow up, were excluded from this analysis (n=1).

DNA sequencing and bioinformatics

This study utilised whole exome sequencing (WES) data of 46 patients with central pubertal delay (i.e. SLDP or IHH), and WES data of 35 healthy control individuals with normal pubertal timing. WES was performed on DNA extracted from peripheral blood leukocytes, using an Agilent V5 platform and Illumina HiSeq 2000 sequencing. Fraction of target regions with coverage >4x was 99.4-99.8%. The exome sequences were aligned to the UCSC hg19 reference genome using the Burrows-Wheeler Aligner software (BWA-MEM [bwa-0.7.12]. Picard tools [picard-tools-1.119] was used to sort alignments and mark PCR duplicates. The genome analysis toolkit (GATK-3.4-46) was used to realign around indels and recalibrate quality scores using

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dbSNP, Mills and 1000 genomes as reference resources. Variant calling and joint genotyping using pedigree information was performed using HaplotypeCaller in GVCF mode from the genome analysis toolkit. The resulting variants were filtered using the variant quality score recalibration (VQSR) function from GATK.

Analysis of the called variants was performed using Ingenuity Variant Analysis (QIAGEN Redwood City, www.qiagen.com/ingenuity). Filtering for potential causal variants was carried out using a classic bioinformatic pipeline (Figure 1). A virtual panel of 47 genes, previously reported in the literature to have a causal role in the pathogenesis of delayed puberty (Table 1), including IHH (13, 18) and SLDP (9, 10, 14, 19-21), was applied as a filter across the whole exome sequencing dataset. Quality control filters included thresholds for call quality, read depth and Phred strand bias, and only variants with minor allele frequency (MAF) <0.5% in the Genome Aggregation Database (gnomAD) database (accessed March 2021) were retained. Predicted functional annotation prioritised nonsense, exonic missense, splice site variants, structural or promoter changes. Case-control analysis identified variants present in affected individuals and not present in controls, using a control group of 35 individuals previously whole exome sequenced using the same bioinformatics pathway, who have been accurately phenotyped as having timing of puberty within the normal range. To identify likely disease-causing variants in these 47 genes, only variants that met the ACMG criteria (22) for pathogenicity, likely pathogenicity, or variants of uncertain significance (VUS) were selected in the analysis.

VUS identified were further selected for being potentially deleterious using *in silico* analysis software tools: SIFT (23), PolyPhen-2 (24), Mutation taster (25), REVEL (26), and MetaLR (27). Variants predicted to be deleterious by ≥ 3 from 5 tools were retained (Figure 1). Promoter

variants were annotated using RegulomeDB (https://regulomedb.org/regulome-search) and Haploreg (https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php) resources.

Genotypic criteria for the diagnosis of IHH

Using the filtered WES data, the genotype of each patient was determined to support a genotypic diagnosis of either SLDP or IHH. This categorisation was done without consideration of clinical data to minimise the risk of bias in genotype interpretation. The genotype interpretation was assisted by bioinformatic tools and published literature. Information pertaining to previously reported mutations, including variant location in the protein structure and inheritance characteristics of variants in each gene, was reviewed. The pattern of inheritance for each variant in both the proband with pubertal delay and family relatives was used to determine whether the variant might cause IHH or SLDP (Table S1).

A genotypic diagnosis of IHH was made if the patient carried: i) a known deleterious variant in a known IHH gene with the same zygosity as previously demonstrated to cause IHH, ii) a new predicted deleterious variant in a known IHH gene with the same zygosity as previously demonstrated to cause IHH, iii) homozygosity for a new predicted deleterious variant where heterozygosity or homozygosity of this gene is reported in IHH.

A genotypic diagnosis of SLDP was made if the patient carried a known or new predicted deleterious variant in a known SLDP gene with the same zygosity as previously demonstrated to cause SLDP.

An inconclusive genotype was called if: i) no variants were found, or ii) a new predicted deleterious variant in a known IHH gene with discordant zygosity to that previously demonstrated e.g. heterozygous where homozygous carriage has been shown to cause IHH, or

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iii) oligogenic carriage of 2 separate predicted deleterious variants in 2 IHH genes or a mix of IHH and SLDP genes were identified.

Ethical approval and consent to participate

Ethical approval was granted by the London-Chelsea NRES committee (13/LO/0257). All participants provided written informed consent prior to study participation. The study was conducted in accordance with the guidelines of The Declaration of Helsinki.

Statistical analysis

Continuous data were expressed as mean and standard deviation (SD) when normally distributed or median with interquartile range (IQR) otherwise. Fisher's exact test was used to compare categorical variables in epidemiological data. Unpaired t-test (two-tail) or, for multiple comparisons, the Mann-Whitney U test, were used to compare continuous variables as appropriate. Cohen's kappa coefficient (κ) was used to demonstrate the correlation between genotypic and clinical diagnoses. Statistical differences were deemed significant at a p-value<0.05. Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software).

Results

Clinical characteristics of SLDP and IHH patient groups are similar at presentation.

From 46 patients presenting at initial assessment with delayed puberty, 54.3% (n=25) had SLDP, and 45.7% (n=21) had IHH as their final clinical diagnosis. The majority were male accounting

for 87% (n=40/46) and gender distributions were not different between SLDP and IHH groups. All clinical details are shown in Table 2. The median age at presentation was 16.0 (IQR 15.0, 17.1) years, with the majority of patients in early puberty, demonstrated by 40% of male patients with ≤ 3 mL and 47.5% with 4-9 mL testicular volumes, 34.8% with prepubertal pubic hair (PH1) Tanner stage and 50% with PH2, and 50% with prepubertal breast (B1) stage and 16.7% with B2 at first clinical assessment. Age at first clinical sign of puberty (achievement of G2 or B2) was 15.6 vs 16.4 years in males and 11.8 vs 16.3 years in females for SLDP and IHH groups, respectively. A family history of pubertal delay was identified in 72% of those in the SLDP final clinical diagnostic group and 47.6% of the IHH group (*p-value=0.2*). Micropenis with bilateral cryptorchidism was found in 2 patients with a final clinical diagnosis of IHH, and of these 2 patients, one also demonstrated synkinesis, whilst a third patient had a cleft palate. Anosmia was found in 5 patients, all in the IHH group (0% vs 25%, *p-value=0.03*); anosmia was therefore present in 25% of IHH patients. All patients had normal basal pituitary function (thyroid function tests, cortisol, IGF1, and prolactin). Thus, most patients in this cohort presented with isolated delayed puberty without clear clinical signs of IHH (e.g. micropenis, cryptorchidism or synkinesis), representative of the DP patients in whom the distinction between SLDP and IHH is considered most difficult.

Multiple deleterious variants in known pubertal delay genes are identified from whole-exome sequencing of patients with delayed puberty

Of the 46 patients analysed by WES, fifteen patients (32.6%) were identified with potentially deleterious variants in a known gene reported in either IHH or SLDP (Figure 2). In all, 15 potentially deleterious variants in 12 genes were identified, with two siblings (patient 13 and 14) carrying the same variant, (*GNRHR* c.317A>G; p.Q106R). All variants were rare with a MAF of

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less than 0.5%, and the majority, 73.3% (n=11/15), were inherited in a heterozygous manner. The exception to this were variants in *GNRHR* (c.317A>G; p.Q106R), *PROKR2* (c.809G>A; p.R270H) and *TAC3* (c.209-1G>C), which displayed homozygous inheritance. One variant was identified in a gene confined to SLDP, *IGSF10* (1 of 4 genes in this category, 25%). Five of the genes reported in both SLDP and IHH were identified with 8 variants in total (5 of 7 genes in this category, 71%), and 6 variants were identified in 6 genes previously reported only in IHH (9 of 35 genes in this category, 17.1%) (Figure 2A). The most common type of allelic variation was missense (63.2%) followed by variants affecting a splice site (15.8%), nonsense (10.6%), promoter (5.3%) and in-frame deletion (5.3%) variants (Figure 2B). The *KISS1R* promoter variant (c.-249G>A) occurred within a region with a highly significant puberty-specific differential promoter methylation pattern (28), predicted to be a site of EZH2 transcription factor binding, a member of the polycomb group of transcriptional regulators (29). The details of each deleterious variant, including zygosity, MAF and predicted functional impact are described in Table 3.

Genotypic characteristics are distinct between IHH and SLDP patient groups.

We compared the genetic characteristics between SLDP and IHH patients. In patients with IHH an underlying genetic variant was identified more frequently (9/21, 42.9%) than in those with SLDP (6/25, 24.0%), but the difference was not significant ($p = 0.2$). Deleterious variants previously reported only in SLDP were not identified in IHH patients. Homozygous, promoter and nonsense variants were identified only in patients with a final clinical diagnosis of IHH. Patients with a clinical diagnosis of SLDP were found to carry potentially deleterious variants in genes previously only reported in IHH, including *DMXL2, OTUD4*, and *SEMA3E* (Table 4).

Genetic criteria can be utilised for the diagnosis of IHH and SLDP in patients presenting with delayed puberty

After a list of qualified variants was filtered, each patient's genotypic diagnosis was determined based on the criteria outlined in methods. One patient was found to have a genotypic diagnosis of SLDP, 7 patients had a genotypic diagnosis of IHH, and the remaining 7 had an inconclusive genotype (Table 5). Notably, 3 patients who were initially given a clinical diagnosis of SLDP (patients 10, 13, and 14) were shown to have a diagnosis of IHH by genetic analysis. At final diagnosis, these 3 patients had a confirmed clinical diagnosis of IHH after completion of follow up. These 3 cases are in keeping with the published literature of these known pathogenic mutations, where cases with IHH can present initially with simple delayed puberty (30, 31). Parents of these cases, as in the literature, were either unaffected carriers or manifested self-limited delayed puberty, Table S3 (30, 32). Autosomal recessive inheritance is a known inheritance pattern in IHH, but phenotypically these cases are at the milder end of the spectrum, not associated classically with red flag signs for IHH, and thus are more likely to be misdiagnosed clinically. Homozygosity in the absence of consanguinity for these families is due, for the *GNRHR*_Q106R variant, by its MAF of 0.4% in the non-Finnish European population, and, for the *TAC3* splice variant to it being a founder mutation in the Congolese population, from where the patient's parents both originated, Table S3.

A correlation between genotypic and clinical diagnosis was identified for patients presenting with delayed puberty

The correlation between final clinical diagnosis and genotype was assessed in 15 patients who were definitively diagnosed with either SLDP or IHH following the diagnostic criteria outlined above. Notably, the patients who were diagnosed with SLDP and IHH by genotyping had SLDP

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and IHH phenotypes, respectively, with a Cohen's kappa (k) = 0.327 (95% IC, 0.137 to 0.517), demonstrating that genotype diagnosis has fair agreement with the final clinical diagnosis (Figure 3). Patients diagnosed as IHH by genotypic criteria are thus very likely to have IHH as a final clinical diagnosis. Likewise, patients with a genotype of SLDP are likely to have a clinical diagnosis of SLDP. For those patients who were diagnosed with an inconclusive genotype by WES, they may belong to the clinical phenotypic group of either SLDP or IHH.

Using this genotypic diagnostic framework, 7 of 21 patients with a final clinical diagnosis of IHH can be diagnosed with IHH, and all patients with a final clinical diagnosis of SLDP had a genetic result which was not compatible with IHH. Applying these criteria resulted in a sensitivity and specificity of 33.3% and 100%, respectively, of using genotypic criteria for diagnosis of IHH, with a positive predictive value (PPV) of 100%, and negative predictive value (NPV) of 64.10% (Table S2).

Discussion

Pubertal delay can be the presentation of a broad spectrum of clinical phenotypes ranging from IHH, which is a pathological condition and needs intensive medical therapy, to SLDP, a more benign condition usually compatible with normal reproductive capacity post puberty. Many clinical and biochemical parameters have been applied to try to distinguish these two conditions; however, these all have limitations in terms of specificity and sensitivity (3). Diagnostic uncertainty is associated with increased psychological stress for both adolescents and their parents (33). In this study, we investigated for the first time whether identification of a genetic defect in patients with pubertal delay, through WES combined with a virtual panel, can distinguish these two conditions.

Prompt diagnosis will aid clinical management, by ameliorating the need for patients to undergo unnecessary investigations or inappropriate treatment. Furthermore, early diagnosis of IHH can facilitate the use of optimal therapeutic modalities for pubertal induction, such as the use of gonadotropins in males with IHH and commencement of therapy at an earlier age than the standard sex steroid therapy indicated for SLDP patients (6). Our clinical data highlight this issue, as the mean age of development of secondary sexual characteristics, following hormonal induction, in the patients with a final diagnosis of IHH was over 16 years.

The findings of this study point to a fair correlation between genotypic diagnosis and final clinical diagnosis, with a 100% specificity and PPV of genetic testing for the diagnosis of patients with IHH. This study also found that patients who carry homozygous or loss of function variants in genes reported in IHH will be very likely to have a final clinical diagnosis of IHH. On the other hand, patients carrying variants reported only in SLDP with heterozygous inheritance are likely to have a clinical diagnosis of SLDP. From this cohort review we found that testing through WES with a virtual panel can make a definitive diagnosis for 17.4% (n=8/46) of patients presenting with pubertal delay, and that of these patients, 7 out of 8 had IHH. This supports the use of genetic investigation in the clinical setting, combined with clinical and biochemical criteria, to confirm the diagnosis of IHH in adolescents presenting with pubertal delay.

Moreover, genetic testing also has benefit for initial diagnosis in patients who do not manifest obvious clinical signs of IHH, as we identified a genotypic diagnosis of IHH in 3 patients in whom there had been an initial clinical diagnosis of SLDP, who went on to have a final clinical diagnosis of IHH. In these patients, who presented with isolated DP without red flags for IHH, where there was also a family history of DP, a default diagnosis of SLDP was made. Such individuals may also have reasonable pre-pubertal testes volumes (e.g. 3mls) or have entered

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puberty and then arrested, and without genetic analysis it is only after several years of careful follow up that they can be diagnosed as IHH.

This study also identified deleterious variants in three other genes known to contribute to IHH, namely *DMXL2, OTUD4* and *SEMA3E,* in 3 patients with a final clinical diagnosis of SLDP. Although these patients had clinical characteristics of SLDP, they may have a more significant defect in their GnRH neuroendocrine system, including impact on fertility or timing of menopause/andropause, which may need monitoring into adulthood. Moreover, it suggests that in a subset of patients with pubertal delay there may be some overlap of genetic and pathophysiological mechanisms between SLDP and IHH, or lie along a spectrum of GnRH deficiency. This sub-category may be reflected by the 'inconclusive genotypic' group identified in this study, in whom a moderate burden of mutations in GnRH deficiency genes may lead to a SLDP phenotypic pattern, whilst a severe mutational burden, such as homozygous or loss of function mutation, may lead to a more severe reproductive phenotype, i.e. IHH. It is likely that with further genetic discovery and better understanding of the pathophysiology underlying these conditions, the genotype-phenotype correlation in this inconclusive group will become clearer. We believe that this group merits careful observation of their clinical progression.

Our study has some limitations. The number of genes in the virtual panel associated with SLDP (n=4) was far smaller than those associated with IHH (n=42). Only a few causal genes have been identified in SLDP to date, leading to a lower pick-up rate for SLDP mutations in this study (deleterious variants were identified in 25% [1 in 4] SLDP genes and 33% [14 in 43] of IHH genes in this study). Therefore, our group is working to better characterise the genetic basis of SLDP. Identification of a larger number of SLDP genes and their use in the virtual panel will improve the ability to make a genetic diagnosis in SLDP patients at presentation. Moreover,

genotypic interpretation in patients with oligogenic inheritance is complex because of our lack of knowledge of variant-variant interaction. We have described such patients' genotypes as inconclusive to minimize potential bias. Furthermore, although some patients had a family history of DP, DNA from the majority of these family members was not available to enable analysis of genotype-phenotype correlation in the wider pedigree. Finally, this genetic analysis shows lower sensitivity and negative predictive value than some biochemical modalities (3, 34-38). Given that a combination of investigations can increase the sensitivity and specificity to diagnosis IHH (39, 40), this type of genetic analysis is at present likely to be best combined with biochemical profiling (e.g. basal LH, FSH, inhibin B, AMH) in order to maximise the diagnostic accuracy.

To our knowledge, this is the first study to demonstrate the correlation between genotypic diagnosis and final clinical diagnosis in a cohort of adolescent patients with severe pubertal delay, validating the use of genetic analysis to support the distinction between the clinical diagnosis of SLDP and IHH. We have also described a set of genotypic criteria for interpreting WES results from a virtual panel using curated information from previous reports. Use of early genetic diagnosis in this condition has the potential for significant cost savings as it can prevent unnecessary investigations and lead to improved health and fertility outcomes for patients. In summary, our analysis shows that WES analysis using a virtual panel in patients with delayed puberty is a useful tool to give a definite diagnosis in an uncertain clinical presentation.

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Accepted Manuscript published as EJE-21-0387.R1. Accepted for publication: 17-Aug-2021

Figure Legends

Figure 1. The analytic pipeline used for identifying genetic defects in patients with pubertal delay, using known genes reported in IHH or SLDP; MAF: minor allele frequency, DP: delayed puberty.

Figure 2. Variants identified in this cohort A) Proportion of variants identified in each gene group; yellow indicates genes confined to SLDP; blue, variants in genes reported in IHH only; and grey indicated variants in genes reported in both SLDP and IHH. B) Number of alleles in each variant category

Figure 3. Clinical diagnosis of the patients who underwent WES with variants identified grouped by genotypic criteria (n=19). X-axis shows 3 groups of genotypic diagnosis in patients underwent WES. Y-axis shows the percentage of patients in each genotype diagnosis. Clinical diagnosis is shown by bars as indicated.

Table 1. List of the genes previously reported causing IHH and SLDP used as a virtual panel during WES analysis (9, 10, 12-14, 18-21, 41)

		n	**Genes**
Genes reported in			
	IHH	36	*ANOS1, CCDC141, DCC/NTN1, DMXL2, FEZF1, FGF17, FGF8, FGFR1, FSHB, GNRH1, KISS1, KISS1R, KLB, LEP, LEPR, LHB, NSMF, NR0B1, NTN1, OTUD4, PCSK1, PLXNA1, PNPLA6, POLR3A/B, PROK2, PROKR2, RNF216, SEMA3E, SMCHD1, SOX10, SOX2, STUB1, TUBB3, WDR11*
	IHH and SLDP	7	*HS6ST1, GNRHR, IL17RD, TAC3, TACR3, SEMA3A, CHD7*
	SLDP	4	*EAP1, IGSF10, LGR4, FTO*

 Accepted Manuscript published as EJE-21-0387.R1. Accepted for publication: 17-Aug-2021

Table 2 Demographic data and clinical details of pubertal delay patients both from the total cohort and compared between final clinical diagnosis of IHH and SLDP. *First visit data was missing from 1 female with IHH; ǂdata presented as median (IQR), otherwise presented as mean (SD). BMI: body mass index, DP: delayed puberty MPH: mid parental height, SDS: standard deviation score, S-FSH: serum follicle-stimulating hormone, S-LH: serum luteinizing hormone, S-Test: serum testosterone, TV: testicular volume, BII/GII: Tanner stage II

	Total (n=46)		**Final Clinical Diagnosis**		***p-value***
			SLDP (n=25)	**IHH (n=21)**	
Patients with identified variants, n (%)	15 (32.6)		6 (24.0)	9 (42.9)	0.2
Gender, n (%)					0.1
Male	40 (87.0)		24 (96.0)	16 (76.2)	
Female	6 (13.0)		1 (4.0)	5 (23.8)	
Ethnicity					0.5
European	29		16	13	
South Asian	6		2	4	
Ashkenazi Jewish	5		5	0	
Middle Eastern	2		2	0	
Turkish	2		0	2	
African	2		0	2	
Age at Tanner stage II (years)					
Male	15.9 (1.5)		15.6 (1.4)	16.4 (1.6)	0.1
Female	15.4 (2.3)		11.8 (0.0)	16.3 (1.2)	0.03*
Hormonal treatment, n (%)	40 (87.0)		21 (84.0)	19 (90.5)	0.8
Anosmia, n (%)	5 (10.9)		0 (0.0)	5 (23.8)	0.04*
MPH	173.4 (7.9)		173.5 (7.77)	173.3 (8.44)	0.9
MPH SDS	-0.41 (0.93)		-0.48 (1.06)	-0.30 (0.73)	0.6
Family history of DP, n (%)	28 (60.9)		18 (72.0)	10 (47.6)	0.2

Consanguinity, n (%)	1 (2.2)		0 (0.0)	1 (4.8)	0.9
IHH red flag signs, n (%)	2 (6.5)		0 (0.0)	3 (14.3)	0.9
First visit *					
Age (years) ǂ	16.0 [15.0, 17.1]		15.6 [14.9, 16.3]	16.5 [15.7, 17.2]	0.05
Weight SDS	-1.19 (1.78)		-1.58 (1.79)	-0.69 (1.69)	0.1
Height SDS	-1.67 (1.15)		-1.80 (1.10)	-1.51 (1.22)	0.4
BMI SDS	-0.29 (1.87)		-0.67 (1.79)	0.18 (1.90)	0.139
Tanner staging					
TV (mL), n (%)					0.2
≤ 3 mL	16 (40.0)		7 (29.2)	9 (56.2)	
4-9 mL	19 (47.5)		14 (58.3)	5 (31.2)	
10-15 mL	5 (12.5)		3 (12.5)	2 (12.6)	
Genital staging, n (%)					0.1
I	16 (40.0)		11 (45.8)	5 (31.2)	
II	16 (40.0)		6 (25.0)	10 (62.5)	
III	5 (12.5)		5 (20.8)	0 (0.0)	
IV	2 (5.0)		1 (4.2)	1 (6.2)	
V	1 (2.5)		1 (4.2)	0 (0.0)	
Pubic hair staging, n (%)					0.3
I	16 (34.8)		9 (36.0)	7 (35.0)	
II	23 (50.0)		14 (56.0)	9 (45.0)	
III	3 (6.5)		0 (0.0)	3 (15.0)	
IV	1 (2.2)		1 (4.0)	0 (0.0)	
V	3 (6.5)		1 (4.0)	1 (5.0)	
Breast staging, n (%)					0.8
I	3 (60.0)		1 (100.0)	2 (50.0)	
II	1 (20.0)		0 (0.0)	1 (25.0)	
III	1 (20.0)		0 (0.0)	1 (25.0)	
S-LH (IU/L) ǂ	0.8 [0.2, 1.8]		0.8 [0.2, 2.1]	1.0 [0.1, 1.6]	0.6
S-FSH (IU/L) ǂ	1.8 [1.1, 2.8]		2.0 [1.6, 3.4]	1.5 [0.7, 2.7]	0.07
S-Test (nmol/L) ǂ	0.7 [0.4, 1.5]		1.0 [0.6, 4.9]	0.4 [0.4, 0.7]	0.007*
Peak S-LH (IU/L) ǂ	9.1 [1.7, 13.3]		11.70 [8.12, 14.25]	6.00 [1.17, 10.72]	0.09
Inhibin B (pg/mL) ǂ	51.0 [18.5, 115.0]		149.0 [104.0, 183.0]	36.0 [14.5, 69.8]	0.009*

Accepted Manuscript published as EJE-21-0387.R1. Accepted for publication: 17-Aug-2021

Table 3. Identified variants in pubertal delay patients. X indicates a potentially deleterious variant by prediction tools (deleterious by SIFT, probably or possibly damaging by PolyPhen2, disease causing by Mutation Taster, likely disease causing by REVEL, and damaging by MetaLR); O indicates non-pathogenic predicted by prediction tools (tolerated by SIFT, benign or unknown by PolyPhen2, polymorphism by Mutation Taster, likely benign by REVEL, and tolerated by MetaLR), Het; heterozygous, Hom; homozygous, P; pathogenic, LP; likely pathogenic, VUS; variant of uncertain significance

Gene lists/ Genes	**Variants**	**Protein variants**	**Reference**	**Translational impact**	**Zygosity**	**ACMG crtieria**	**SIFT**	**PolyPhen -2**	**Mutation taster**	**REVEL**	**MetaLR**
SLDP only											
IGSF10	c.7124A>G	p.N2375S		Missense	Het	**VUS**	**X**	**X**	**X**	**X**	**X**
Both SLDP & IHH											
GNRHR											
	c.317A>G	p.Q106R	(42) , (43)	Missense	Hom	**P**	**X**	**X**	**X**	**X**	**O**
	c.436C>T	p.P146S	(44)	Missense	Het	**LP**	**X**	**X**	**X**	**X**	**X**
SEMA3A	c.1849C>T	p.R617Ter		Nonsense	Het	**P**	**-**	**-**	**X**	**-**	**-**
TAC3											
	c.209-1G>C	-	(31)	Splice site	Hom	**P**	**-**	**-**	**X**	**-**	**-**
	c.*2-1G>T	-		Splice site	Het	**LP**	**-**	**-**	**-**	**-**	**-**
TACR3	c.1090C>T	p.R364Ter		Nonsense	Het	**P**	**-**	**-**	**X**	**-**	**-**

Accepted Manuscript published as EJE-21-0387.R1. Accepted for publication: 17-Aug-2021

	CHD7	c.3738G>A	p.M1246I		Missense	Het	**VUS**	**X**	**X**	**X**	**X**	**X**
IHH only												
	DCC	c.1933C>T	p.P645S	(45)	Missense	Het	**VUS**	**X**	**X**	**X**	**O**	**O**
	DMXL2	c.2540C>T	p.T847I		Missense	Het	**VUS**	**X**	**X**	**X**	**O**	**O**
	KISS1R	c.-249G>A	-		Promoter	Het	**VUS**	-	-	-	-	-
	OTUD4	c.458_460delCTG	p.A153del		In-frame deletion	Het	**VUS**	-	-	-	-	-
	PROKR2	c.809G>A	p.R270H	(46)	Missense	Hom	**VUS**	**X**	**X**	**X**	**X**	**X**
	SEMA3E	c.398G>T	p.C133F		Missense	Het	**VUS**	**X**	**X**	**X**	**X**	**X**

Table 4. Comparison of genetic characteristics between patients clinically diagnosed as SLDP and IHH

	Final Clinical Diagnosis	
	SLDP (%)	**IHH (%)**
Patient with variant identified		
Yes	6 (24.0)	9 (42.9)
No	19 (76.0)	12 (57.1)
Variants from gene list		
SLDP only	1 (14.2)	0 (0.0)
SLDP & IHH	2 (42.9)	6 (66.7)
IHH only	3 (42.9)	3 (33.3)
Zygosity of the variants		
Heterozygous	6 (100.0)	5 (55.6)
Homozygous	0 (0.0)	4 (44.4)
Variant category (n=20 alleles)		
Predicted to affect protein structure/ expression (nonsense, splice site, promoter)	1 (16.7)	5 (38.5)
Predicted not to affect protein structure/ expression (missense, in-frame variant)	5 (83.3)	8 (61.5)

Accepted Manuscript published as EJE-21-0387.R1. Accepted for publication: 17-Aug-2021

Table 5. The association between final clinical and genotypic diagnosis. Het: heterozygous, Hom: homozygous. Genotypic diagnosis shown in bold, where discordant with initial clinical diagnosis but concordant with final clinical diagnosis.

No.	Initial Clinical Diagnosis	Final Clinical Diagnosis	Sex	Age at 1st visit (yrs)	DP in family	Gene	Ethnicity	Nucleotide Change	Protein Change	MAF (%) by ethnicity	Zygosity	Genotypic Diagnosis
1	Uncertain	SLDP	M	18.3	No	*IGSF10*	Non-Finnish European	c.7124A>G	p.N2375S	0.0256	Het	SLDP
2	Uncertain	SLDP	M	13.3	No	*DMXL2*	Askenazi Jewish	c.2540C>T	p.T847I	0	Het	Inconclusive
3	Uncertain	SLDP	M	17.2	No	*OTUD4*	Non-Finnish European	c.458_460delCTG	p.A153del	0.00558	Het	Inconclusive
4	Uncertain	SLDP	M	17.4	No	*CHD7*	Non-Finnish European	c.3738G>A	p.M1246I	0	Het	Inconclusive
5	Uncertain	SLDP	M	17.2	Father, uncle	*SEMA3E*	Non-Finnish European	c.398G>T	p.C133F	0	Het	Inconclusive
6	Uncertain	SLDP	M	15.0	Father, uncle	*TAC3*	African	c.*2-1G>T	Splice site	0.0259	Het	Inconclusive

Accepted Manuscript published as EJE-21-0387.R1. Accepted for publication: 17-Aug-2021

7	Uncertain	IHH	M	15.6	Father	*PROKR2*	South Asian	c.809G>A	p.R270H	0.0588	Hom	IHH
8	Uncertain	IHH	M	14.8	Mother	*KISS1R*	Non-Finnish European	c.-249G>A	Promoter	0	Het	Inconclusive
9	Uncertain	IHH	M	15.9	Parents, brother	*SEMA3A*	Non-Finnish European	c.1849C>T	p.R617Ter	0.00088	Het	IHH
10	SLDP	IHH	F	14.9	Mother, sister	*TAC3*	African	c.209-1G>C	Splice site	0.112	Hom	**IHH**
11	Uncertain	IHH	M	18.1	Father	*GNRHR*	Non-Finnish European	c.436C>T	p.P146S	0.119	Het	Inconclusive
12	Uncertain	IHH	M	17.1	No	*DCC*	South Asian	c.1933C>T	p.P645S	0.173	Het	IHH
13	SLDP	IHH	M	16.5	Mother, brother	*GNRHR*	Non-Finnish European	c.317A>G	p.Q106R	0.418	Hom	**IHH**
14	SLDP	IHH	M	13.5	Mother, brother	*GNRHR*	Non-Finnish European	c.317A>G	p.Q106R	0.418	Hom	**IHH**
15	Uncertain	IHH	M	16	No	*TACR3*	South Asian	c.1090C>T	p.R364Ter	0.00327	Het	IHH

Accepted Manuscript published as EJE-21-0387.R1. Accepted for publication: 17-Aug-2021

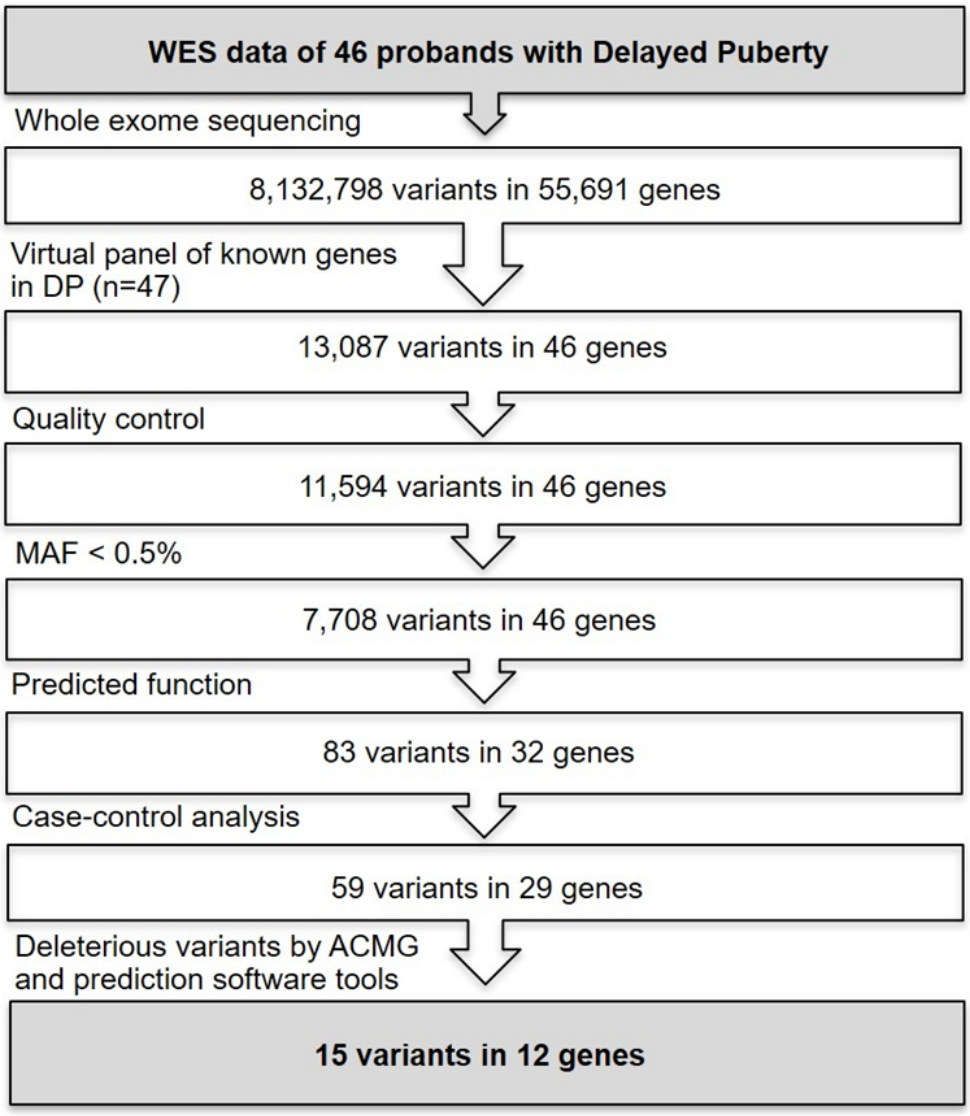

Figure 1. The analytic pipeline used for identifying genetic defects in patients with pubertal delay by using known genes reported in IHH or SLDP; MAF: minor allele frequency, DP: delayed puberty.

130x145mm (150 x 150 DPI)

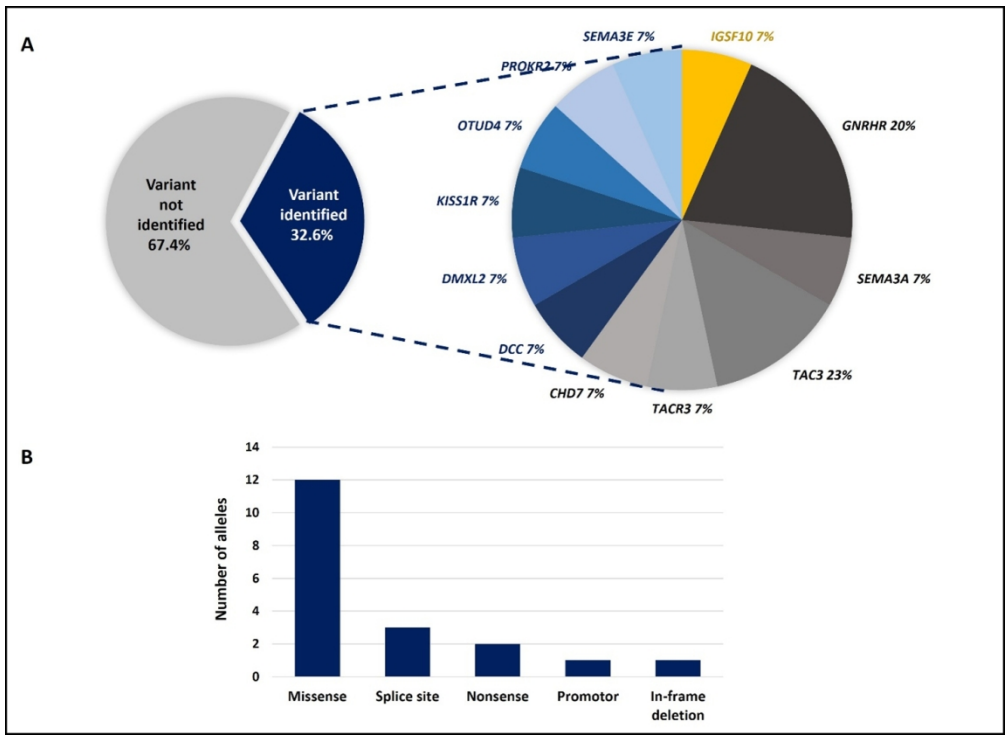

Figure 2. Variants identified in this cohort A) Proportion of variants identified in each gene group; yellow indicates genes confined to SLDP; blue, variants in genes reported in IHH only; and grey indicated variants in genes reported in both SLDP and IHH. B) Number of alleles in each variant category

169x123mm (220 x 220 DPI)

Accepted Manuscript published as EJE-21-0387.R1. Accepted for publication: 17-Aug-2021

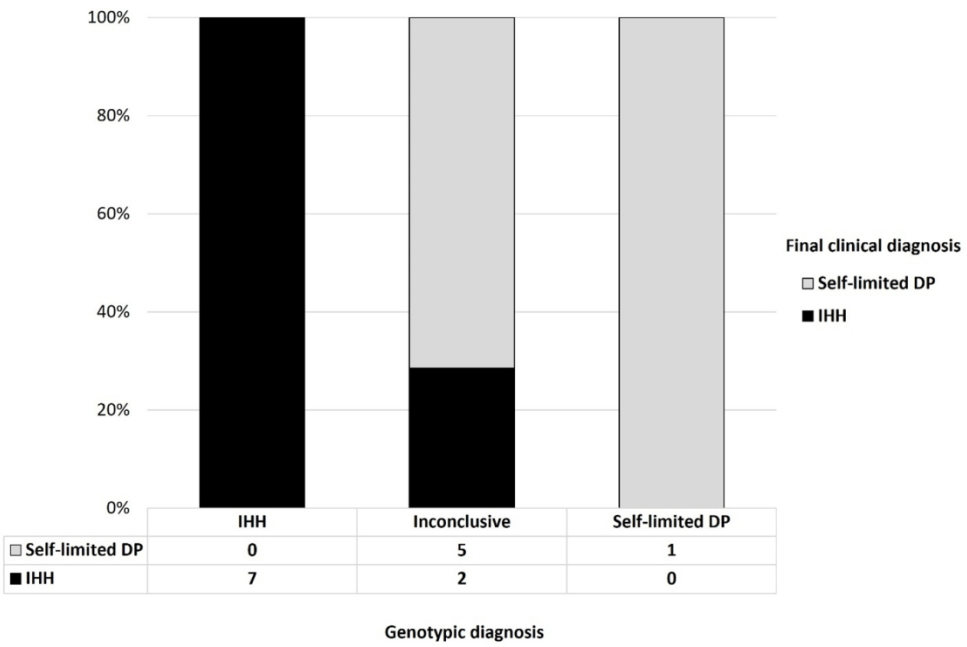

Figure 3. Clinical diagnosis of the patients who underwent WES with variants identified grouped by genotypic criteria (n=15). X-axis shows 3 groups of genotypic diagnosis in patients underwent WES. Y-axis shows the percentage of patients in each genotype diagnosis. Clinical diagnosis is shown by bars as indicated.

167x110mm (220 x 220 DPI)

Table S1 Genotype diagnosis by variant using literature review of each gene and variant that have been previously reported in IHH/KS, self-limited DP and unaffected individuals. Het: heterozygous, Hom: homozygous, KS: Kallmann syndrome, ID: Intellectual disability, SLDP: self-limited DP, SNHL: sensorineural hearing loss

Genes	Associated phenotype	IHH	SLDP	Normal	Domain disease causing	Variants	Protein variants	Zygosity	Variant lies in specific-domain	Variant specific for phenotype	Genotype Dx
CHD7 (1-4)	CHARGE features	Het, oligogenic het	Het	Het	Mutations were found along the protein in both specific and non-specific domains	c.3738G>A	p.M1246I	Het	Non-specific	No	Inconclusive
DCC (5, 6)	Facial asymmetry, MR, obesity, SNHL	Het, oligogenic het	No	Het	5 mutations have been reported which are located in specific domains including Ig-like C2 domain type 2 and fibronectin type III domain 1, 3, 5	c.1933C>T	p.P645S	Het	Fibronectin type III domain 3	p.P645S (het) has KS	IHH

Accepted Manuscript published as EJE-21-0387.R1. Accepted for publication: 17-Aug-2021

DMXL2 (7, 8)	none- autoimmune DM, demyelinating polyneuropathy, MR	Hom	No	Het	3 cases with homozygous deletion of 5 amino acid in exon 24 (c.5824_5838del, p.1942_1946del), which is in non-specific domain, have IHH - Parents and a sister who carry heterozygous of the above mutation have normal pubertal timing	c.2540C>T	p.T847I	Het	non- specific domain	No	Inconclusive
GNRHR (9-14)	-	Hom, compound het	Het	Het	R262Q (hom) has been reported in CDGP	c.317A>G	p.Q106R	Hom	Extracellular loop domain2	-Patients with compound het of p.Q106R and other mutation in GnRH gene have been found to cause IHH - Parent of proband who carries p.Q106R (het) has normal puberty	IHH
						c.436C>T	p.P146S	Het	Intracellular loop domain2	- 2 sisters carry p.P146S (het) have IHH - Mother of 2 sisters carries same mutation	Inconclusive
IGSF10 (15)	-	No	Het, digenic het	Het	2 het mutations in leucine-rich repeats (LRR) domains 1-7 have been reported to cause self-limited DP	c.7124A>G	p.N2375S	Het	Ig domain	No	SLDP

KISS1R (16-19)	-	Het, hom, compound het	Het	Het	More than 30 mutations have been reported to cause DP. The zygosity includes heterozygous, homozygous or compound heterozygous mutation. - Some variants in heterozygous state such as p.A287E, p.P476R do not cause IHH.	c.-249G>A	-	Het	96 base pairs before transcription start	No	Inconclusive
OTUD4 (20, 21)	Progressive dementia & ataxia	Digenic hom	No	Het	- Digenic hom in RNF216 and OTUD4 cause IHH - Individuals with het mutation are unaffected	c.458_460delCTG	p.A153del	Het	Within catalytic domain	No	Inconclusive
PROKR2 (22-27)	-	Hom, digenic het & het	No	no	Most mutations cause IHH/KS	c.809G>A	p.R270H	Hom	3rd intracellular loop domain	-This variant (het) with *SEMA3A* p.R617Q (het) has been found with IHH phenotype	IHH
SEMA3A (28-30)	-	Het, oligogenic het	No	No	Heterozygous mutation of *SEMA3A* located along the gene have been found to cause IHH/KS.	c.1849C>T	p.R617Ter	Het	Ig domain	p.R617Q mutation combined with heterozygous mutation of *CCDC141* and *PROKR2* was found in IHH.	IHH
SEMA3E (31, 32)	-	Oligogenic het	No	No	Oligogenic het mutation in SEMA and Ig domain have been found in IHH/KS.	c.398G>T	p.C133F	Het	SEMA domain	Mutation in the same domain, p.M102T, has been found in KS when combine with heterozygous mutations of	Inconclusive

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										PLNXA1 and *CCDC141*.	
TAC3 (33-38)	-	Hom, digenic het	Het	No	Patient with IHH has homozygous or digenic heterozygous mutation of this gene. - This gene is also found in family members of probands with IHH. The reported mutations are missense and mutation in splice site of TAC3.	c.209-1G>C		Hom	-	Heterozygous splice site mutation (TAC3 g.18595G>T) has been reported in individuals with delayed puberty; homozygous carriage of this variant has been reported in individuals with IHH with reversal	IHH
						c.*2-1G>T		Het	-		SLDP
TACR3 (33, 35-38)	-	Hom, compound het, het	Het	Het	- Mutation in this gene have been found in both IHH and self-limited DP. Heterozygous mutation of this gene can cause both conditions.	c.1090C>T	p.R364Ter	Het	Intracellular region	No	IHH

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Table S2. 2X2 table for analysis of the utility of genotypic diagnosis to diagnosis IHH in patients with pubertal delay (n=46)

		Final clinical diagnosis	
		IHH (n=21)	**SLDP (n=25)**
Genotypic diagnosis	**IHH**	7	0
	Non-IHH	14	25

Table S3. Clinical data for family members of probands 10, 13 and 14

Proband 10						
Proband Genotype	Minor allele frequency	Maternal phenotype	Maternal genotype	Paternal phenotype	Paternal genotype	Consanguinity
TAC3_ c.209-1G>C Homozygous	0.1% African/ African American; known founder mutation in the Congolese population (1)	Delayed puberty, AAM 15.5 yrs	TAC3_ c.209-1G>C Heterozygous	Not available	Not available	Not known, but parents both from families originally from the same geographical region of Congo
Proband 13 & 14						
Genotype	Minor allele frequency	Maternal phenotype	Maternal genotype	Paternal phenotype	Paternal genotype	Consanguinity
GNRHR_ c.317A>G_p.Q106R Homozygous	0.4% Non-European Finnish	Delayed puberty, B2 14 yrs, AAM 16 yrs	GNRHR_ c.317A>G_ p.Q106R Heterozygous	Self-recalled normal age of puberty onset	GNRHR_ c.317A>G_ p.Q106R Heterozygous	Nil

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