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特發性低促性腺激素性腺功能低下症臨床和基因方面探討

The clinical and genetic landscape of idiopathic hypogonadotropic hypogonadism

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中文摘要

<簡介>

特發性低促性腺激素性腺功能低下症(Idiopathic hypogonadotropic hypogonadism, IHH)是指下視丘至腦垂體結構正常，病患除促性腺激素低下，其他腦垂體賀爾蒙功能正常，導致其第二性徵發育不全，性腺功能低下。這類病人臨床表現通常有出生時陰莖短小、隱睪、青春期延遲或缺失，例如:女性沒有乳房發育或者無月經等。而根據有無合併嗅覺異常與否分成兩類：嗅覺喪失的卡門氏症候群(Kallmann syndrome)和嗅覺正常特發性低促性腺激素性腺功能低下症(normosmic IHH，nIHH)。

<實驗方法>

我們收集了 33 位診斷 IHH 病人，並分析其表徵、性腺賀爾蒙、骨齡和身高等臨床相關參數和實驗數據，並且利用全外顯子定序(whole exome sequencing)技術分析這些病人基因變異，以美國醫學遺傳學暨基因體學學會(ACMG)遺傳變異分類標準與指南尋找病患致病或可能致病基因。若未找出致病原因之病患，我們先探討寡基因變異（oligogenic variants）可能性和嘗試以拷貝數變異 (copy number variants)偵測基因的局部缺失或增加等分析工具，期找出可能的致病基因。仍找不到任何不明確的變異 (variants of uncertain significance)則納入全基因定序(whole genome sequencing)和 RNA 定序(RNA sequencing)來進一步確認病人可能的致病點位。

<結果>

本研究 33 位病人之中，20 位找到致病或可能致病變異相關基因，其檢出率達 60 %，其中 *FGFR1*(n=6)、*CHD7* (n=5)、*ANOS1*(n=4) 是找到最常見的致病基因之一。4 位病人有不確定的致病基因，進一步分析後，其中 3 位可能存在雙基因或者寡基因變異。9 位病人在外顯子定序檢測後未找到可能的基因變異者，其中 4 位進一步接受全基因及 RNA 定序檢查，1 位病人在全基因定序中發現一個 VUS 點位。

<結論>

完整的基因分析可以幫助我們更及早診斷 IHH 病人，而且經由其致病基因相關綜合症 IHH(syndromic IHH)協助病人做更完整的身體評估和檢查。另外，可以藉由一些基因檢測工具來彌補全外顯子分析的缺點來更加準確知道可能的致病基

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因。此項實驗有助於 IHH 相關基因的發現及了解其調控機制與致病機轉，甚至提供往後更完整檢驗和可能有效的治療方針。

關鍵詞：特發性低促性腺激素性腺功能低下症、卡門氏症候群、全外顯子定序、全基因定序、RNA 定序

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英文摘要(Abstract)

Backgrounds: IHH is a rare condition characterized by gonadal failure due to deficiencies in the production, secretion, and activity of GnRH, with normal levels of other pituitary hormones and no anatomical abnormalities in the hypothalamus-pituitary axis. IHH is further divided into two subcategories: Kallmann syndrome (KS) and normosmic IHH (nIHH). Notably, there is currently no published clinical or genetic data on IHH patients in Taiwan.

Methods: We applied whole exome sequencing (WES) to analysis our 33 IHH patients. Additionally, we categorized the phenotype of these patients, along with their hormone data and height outcomes after undergoing hormone replacement therapy. We also do further whole genome sequencing (WGS) and RNA-sequencing (RNA-seq) for those with uncertain variants and unknown variants.

Results: The study revealed that 20 of them exhibited pathogenic/likely pathogenic (P/LP) variants of IHH-associated genes, yielding a mutation detection rate of 60%. Among these patients, *FGFR1*(n=6), *CHD7*(n=5) and *ANOS1* (n=4) were the most frequent genetic causes of IHH. Nonetheless, we still encountered 13 patients who did not exhibit possible variants of the genes, including a group with variants of uncertain significance and variants (n=4) and a genetically unresolved group (n=9).

Additionally, we remain committed to expanding our understanding of this condition and analysis them by cutting-edge tools and techniques, such as digenic/oligogenic variant prediction, copy number variant analysis, RNA-Seq, and WGS. Only four patients were included in our RNA-seq and WGS data collection, and among them, 1 patient was identified with a variant of uncertain significance (VUS).

Conclusion: To sum up, this study effectively determined the genetic origins of the majority (60%) of IHH cases, with a greater occurrence found among male patients.

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Utilizing exome sequencing combined with targeted analysis of IHH-related genes was a successful approach to uncovering the genes for the disease, both known and unknown. Further cases are needed to provide additional evidence for the effectiveness of RNA-seq and WGS in IHH patients.

Key words: Idiopathic hypogonadotropic hypogonadism, Kallmann syndrome, whole exome sequencing, whole genome sequencing, RNA sequencing

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Chapter 1. Main Contents

1.1Introduction

Definition

The hypothalamic-pituitary-gonadal (HPG) axis is a unique system responsible for pulsatile secretion of GnRH, which occurs during infancy and puberty. Idiopathic hypogonadotropic hypogonadism (IHH) is a rare disorder that leads to gonadal failure due to a deficiency in the production, secretion, and action of GnRH. However, patients with IHH have normal levels of other pituitary hormones, and there are no anatomical defects in the hypothalamus-pituitary axis[1].

Embryology and Classification

IHH can be classified into two types: Kallmann syndrome (KS) and normosmic IHH (nIHH). GnRH neurons, responsible for hormone signaling in the hypothalamic-pituitary-gonadal (HPG) axis, originate in the nasal placode along with olfactory sensory neurons and olfactory ensheathing cells. Improper development or migration of GnRH neurons from the vomeronasal organ to the brain leads to hypogonadism, including KS, which is characterized by anosmia and impaired sexual development[2].

Prevalence and Genetic Contributions

The prevalence of IHH is around 1 to 10 cases per 100,000 births, and KS accounts for 60% of these cases[1]. Approximately 30% to 50% of IHH cases are monogenic, and many candidate genes of different types of inheritance have been described, as well as imprinting loci. Some studies suggest that digenic or oligogenic inheritance may play a role in 10% to 20% of IHH cases[3,4]. The diversity in etiology explains the variation in phenotype and penetrance. Sanger sequencing and targeted next-generation sequencing

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have been used to detect variants of candidate genes involved in hypothalamic and pituitary development, GnRH and olfactory neuron migration, and GnRH synthesis and secretion pathways[5]. However, the cause of IHH remains unknown in many patients[5].

Genetic Diagnosis: Unveiling the Role in IHH

The identification of possible pathogenic genes in patients with IHH can contribute to the diagnosis of associated syndromic diseases. One example is CHARGE syndrome, which is associated with variants in the *CHD7* gene and exhibits a wide range of clinical phenotypes. Moreover, the crucial rationale behind identifying potential pathogenic gene variants in these patients lies in the early differentiation between individuals with IHH and those with constitutional delay of growth and puberty (CDGP). This distinction is of utmost significance since previous investigations on CDGP have resulted in minimal genetic findings, with only a 5% detection rate. This emphasizes the need to identify genetic markers specific to IHH, which has a detection rate of around 50%[6]. Additionally, it is worth noting that a sustained reversal of normosmic idiopathic hypogonadotropic hypogonadism (nIHH) and Kallmann syndrome (KS) was observed in approximately 10% of patients with either absent or partial puberty upon discontinuation of treatment. This intriguing finding suggests a potential association with specific gene variants, including *FGFR1* and *GNRHR*[7].

Treatments of IHH

Patients with IHH not only experience incomplete or absent secondary sexual characteristics and infertility but also exhibit height deficit because of hormone deficiency. In male adolescents, the primary therapeutic strategy for initiating secondary sexual development entails the administration of testosterone esters, such as testosterone

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enanthate or cypionate, via intramuscular injections at 4-week intervals. In females with IHH, oral or transdermal estrogen therapy, particularly in the form of patches, is recommended for pubertal induction in individuals requiring feminization. It is initiated at low doses to replicate early puberty estrogen levels and gradually increased over 12 to 24 months. Progestogen is added after achieving optimal breast development or managing breakthrough bleeding. The hormone replacement treatments are effective in promoting the development of secondary sexual characteristics and height spurts in both males and females[9]. For those of childbearing age, various treatment options can be considered to induce fertility. These include pulsatile GnRH treatment, hCG monotherapy, or a combination therapy involving FSH and hCG[8].

The purpose of our study

The primary objective of this study was to examine the diagnosis and management approaches employed for patients diagnosed with IHH in Taiwan. Furthermore, given the lack of published clinical data regarding the relationship between genotypes and treatment outcomes, our aim was to explore this crucial aspect in order to enhance our understanding and inform more effective therapeutic strategies. Whole exome sequencing (WES) was conducted, focusing on the targeted analysis of 283 genes associated with Idiopathic Hypogonadotropic Hypogonadism (IHH). Also, we further use whole genome sequencing (WGS) and RNA sequencing to detect those patients with unknown genetic results. The data obtained from this study may provide valuable information for the diagnosis, treatment, and genetic counseling of IHH patients in the future.

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1.2 Materials and methods

Subjects

The National Taiwan University Hospital's pediatric endocrine clinic diagnosed 43 unrelated patients with IHH over a span of 20 years from 2002 to 2022. To diagnose IHH, the clinic used specific criteria such as (1) clinical signs of incomplete or absent puberty, (2) low levels of sex steroids, and a relative low gonadotropin response to GnRH stimulation, (3) the absence of organic lesions in the hypothalamus-pituitary region detected through MRI. Based on these criteria, patients were classified as KS if they had a history of anosmia and hypoplasia of the olfactory bulb as verified through MRI or tested by Top International Biotech olfactory device (Taiwan version)[10], while those without anosmia were classified as nIHH. Out of the 43 patients diagnosed with IHH, 33 were followed up at the clinic and enrolled in the study, which underwent review and approval by the Institutional Review Board of National Taiwan University Hospital (IRB No. NTUH201801070RINA, No. 202107027RIND). Before participating, all 33 patients provided signed written consent after receiving a detailed explanation of the nature and purpose of the procedures used (Figure 1).

Clinical characteristics and endocrine evaluation

Upon diagnosis, all patients underwent GnRH tests, followed by regular visits every 3 months for evaluation of height, weight, and signs of puberty. Every 6 months, bone age was assessed through the Greulich and Pyle method[11]. To exclude the possibility of organic lesions in the hypothalamic and pituitary regions and to evaluate the development of the olfactory bulbs, a brain MRI was performed after a diagnosis of IHH had been established. Once the diagnosis was confirmed, hormone replacement therapy (HRT) was initiated at the appropriate age and patients were regularly followed

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up at our pediatric endocrine clinic until the end of the study. The IMMULITE"2000 Analyzer (Siemens, Munich, Germany) was used to measure the levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and estradiol (E2). The ARCHITECT 2nd Generation Testosterone assay (Abbott, Chicago, IL, USA) was employed for measuring the levels of testosterone.

Molecular analysis

In order to extract genomic DNA from peripheral blood leukocytes, the QIAamp DNA Mini Kit was utilized following the manufacturer's instructions. For WES (SureSelectXT Target Enrichment System, V6; Agilent, Santa Clara, CA, USA. Before February 2021, the Sureselft kit was used, while after March 2021, the Roche KAPA HyperExome Probes kit was used instead.), a NovaSeq 6000 sequencer (Illumina Inc., San Diego, CA, USA) was employed for 275 bp paired-end sequencing. Following this, sequence reads were aligned to the human reference genome (GRCh38) with the aid of the Burrow-Wheeler Alignment (BWA) program (version 2.2.1)[12] and then deduplicated by PICARD (https://broadin-stitute.github.io/picard/). For single nucleotide polymorphism (SNP) variant calling from the aligned sequence reads, SAMtools 1.16 and the Genome Analysis Toolkit (GATK version 4.1.3.0, https://gatk.broadinstitute.org/hc/en-us) were employed. The GATK HaplotypeCaller was utilized for insertion-deletion variant calling. Also, the annotation of the sequence was conducted using Ensemble Variant Effect Predictor[13], ANNOVAR[14], and Nirvana (https://github.com/Illumina/Nirvana).

The identical pipeline for WGS analysis was executed for WGS analysis. In regard to RNA sequencing, the STAR tool 2.7.10b was utilized for mapping the sequencing reads to a reference transcriptome[15], and subsequently, GATK HaplotypeCaller was

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utilized for calling variants. To perform annotation, ANNOVAR and nirvana were applied.

To conduct a comprehensive analysis of the causative genes in IHH, we first filtered a targeted gene panel of 283 genes. This selection was primarily based on Quaynor's comprehensive article, which provided detailed descriptions and encompassed a majority of the relevant genes identified through an extensive literature review. These genes are implicated in various processes related to hypothalamic and pituitary development, GnRH neuronal migration, GnRH synthesis and secretion, as well as neurological disorders associated with idiopathic hypogonadotropic hypogonadism (IHH) and rare syndromes linked to hypogonadism[16,17.18,19]. Additionally, we selected previously reported IHH-associated genes from the PubMed database (http://www.ncbi.nlm.nih.gov/pubmed/) and the Online Mendelian Inheritance in Man (OMIM) online database (http://www.ncbi.nlm.nih.gov/omim/) (Table 1). If no candidate variants were identified, whole-exome variants were assessed. Variants with allele frequencies greater than 1% in the Taiwan Biobank (TWB) or with maximum minor allele frequencies (MAF) greater than 1% in any of the following databases: the 1000 Genomes Browser (http://browser.1000genomes.org/), NHLBI ESP Exome Variant Server (http://evs.gs.washington.edu/EVS/), and Genome Aggregation Database (gnomAD; http://gnomad. broadinstitute.org/) were eliminated. Known polymorphisms were annotated using dbSNP (http://www.ncbi.nlm.nih.gov/SNP/), and variant pathogenicity was determined using ClinVar (http://www.ncbi.nlm.nih.gov/clinvar/) and the Human Gene Mutation Database (http://www.hgmd.org/). In silico prediction of the probable effects of novel sequence variants was performed using the web-based programs PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and Sorting Intolerant From Tolerant (SIFT; http://sift.jcvi.org/), as well as various tools in ANNOVAR

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(LRT_score, MutationTaster, MutationAssessor, FATHMM, MetaSVM, MetaLR, M-CAP, Fathmm_MKL_coding, and CADD-Phred). In addition, we employed the ORVAL tool (https://orval.ibsquare.be/) to identify potential digenic or oligogenic variants. Furthermore, we assessed copy number variants using the GATK-gCNV open-source tool(https://github.com/broadinstitute/GATK-gCNV-publication/packages), which utilizes read-depth information to detect rare CNVs in genome sequencing data. The categorization of candidate variants was carried out according to the American College of Medical Genetics Laboratory Practice Committee Working Group (ACMG) guidelines, which classified them as pathogenic, likely pathogenic, variants of uncertain significance (VUS), likely benign, or benign. Also, we use varsome-the human genomics community (https://varsome.com/) to validate classification of these patients' variants. To ensure the accuracy of our findings, we conducted Sanger sequencing on both the proband and the parents who were available for testing.

Statistical analyses

The data analysis was performed using Stata software (version 17.0; StataCorp LLC., College Station, Texas, USA). The chi-squared test was employed to compare two categorical variables, while the Wilcoxon rank sum test was used to compare mean values between groups. Statistical significance was defined as a p-value less than 0.05.

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1.3 Results

Clinical characteristics and hormone data of the IHH patients

This study included 33 patients with IHH, comprising of 21 males and 12 females. The mean age at diagnosis was 12.6 ± 1.3 years for boys and 16.5 ± 0.9 years for girls, with no significant difference between the two groups (p=0.096). Among boys, the main reasons for medical consultation were a small penis (n=16, 76%), cryptorchidism (n=8, 38%), delayed puberty (n=17, 81%), and gynecomastia (n=3, 14%). The girls mostly presented with delayed puberty (n=7, 58%), primary amenorrhea (n=7, 58%), and secondary amenorrhea (n=5, 42%). Other clinical findings included facial dysmorphism in four patients, hearing impairment in two, renal agenesis in one, and digital bony defects in one. There were 17 boys (81%) and 7 girls (58%) who did not experience spontaneous puberty. Among them, 2 boys (9%) and 5 girls (42%) experienced arrested spontaneous puberty. Additionally, there were 2 boys who had not yet reached pubertal age (Table 2). Table 3 outlines the endocrine results of the individuals diagnosed with IHH. Our analysis showed no significant differences in the baseline levels of FSH, LH, or testosterone, as well as the peak levels of FSH and LH following GnRH stimulation, between males with nIHH and those with KS ($p > 0.05$). Similarly, no statistically significant differences were observed in the baseline FSH and LH levels, or peak levels of FSH and LH after GnRH stimulation, between males and females with nIHH ($p > 0.05$).

Molecular results

In our analysis of 33 IHH patients, we discovered a total of 18 pathogenic (n=14) or likely pathogenic (n=4) variants in candidate genes from a targeted gene panel (Table 3). The gene most frequently associated with causation was *FGFR1* (n=6), followed by

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CHD7 (n=5), *ANOS1* (n=4), *HS6ST1* (n=1), *PROKR2* (n=1), and *FGF8* (n=1). Among the patients who had *FGFR1* gene variants, half of them were found to have KS, while the remaining individuals were diagnosed with nIHH. Notably, one of the nIHH patients also exhibited digital bony defects. Of the patients with *CHD7* gene variants, three were diagnosed with KS, while the other two had nIHH. Patient 7 presented with various medical issues, including sensorineural hearing impairment, ear deformity, dysgenesis of the olfactory bulb, facial dysmorphism, and was diagnosed with incomplete CHARGE syndrome due to a nonsense variant in *CHD7*. Patient 9 had a frameshift variant resulting in protein truncation, and patient 10 had a missense variant in the same gene, along with Marfan syndrome. Patient 19 had a missense variant in *PROKR2* and was also found to have an incidental Rathke cleft cyst that required surgical removal. In addition, two patients with pathogenic variants in the *SOX11* gene were detected through extended whole-exome sequencing. Overall, we observed a 60% (20/33) detection rate of pathogenic or likely pathogenic variants in this study, with a higher rate seen in males (n=15, 71%) than females (n=5, 42%) (Table 4).

We identified four patients with variants of uncertain significance in candidate genes, three of whom had possible digenic variants via WES. For 9 patients unsolved genetically, four of them performed whole genomes and RNA sequencing and one patient found to have NF1 variants, as shown in Table 5.

Copy-number variants, incomplete penetrance and digenic pattern in IHH-associated genes

We incorporated gCNV tools to verify the presence of genetic copy number variations in the IHH patients. Upon analysis, we discovered that Patient 15 had a significant deletion in the *ANOS1* gene, which was not identifiable through single

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nucleotide variants (SNV) analysis. Initially, the patient denied experiencing anosmia or hyposmia. However, following an olfactory test, hyposmia was confirmed, and the diagnosis was consistent with the *ANOS1* variant. In the remaining 40 % of patients, the results were inconclusive, suggesting that oligogenic inheritance or other types of genetic heterozygosity may have caused IHH in these cases. Thus, we used ORVAL tool to help us predict possible digenic variants and three patients were identified as digenic variants (Table 5, Case 21,22 and 23). If the pathogenic score of a gene pair is greater than 0.5, we can infer that the digenic variants may have the potential to be the causative genes[20]. Additionally, we observed incomplete penetrance in the family of case 6, where her twin brother who also carried the same variant showed normal puberty and hormone levels, consistent with findings from previous studies [21](Figure 2). Some genes may also exhibit incomplete penetrance pattern and variable expressivity, *PROKR2*(Case 19) and *NOTCH1*(Case 24). Genetic modifiers, mosaicism, polygenic factors, and nonsense-mediated decay efficiency are some of the factors that could contribute to the penetrance and expressivity of genes[22].

Outcomes after hormone replacement therapy in patients with IHH

In the context of hormone replacement therapy for male patients with absent or incomplete puberty, testosterone cypionate was utilized as the treatment modality. The induction of puberty involved the administration of 50mg of testosterone cypionate through intramuscular injections monthly. Subsequently, the dosage increased to the full dose in the following two to three years. Further modifications were made based on the individual's clinical presentation and hormone profile, with the dosage titrated to 200mg per month or administered biweekly. In a similar vein, female patients with absent or incomplete puberty underwent induction therapy utilizing estradiol. The low starting dose of estrogen therapy, for puberty induction and breast development, increased

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gradually over one to two years. Afterward, progesterone, usually as medroxyprogesterone, was added ten days monthly for withdrawal bleeding and uterine development.

Excluded three boys and two girls who had not yet reached their final height, a total of 28 patients (18 males and 10 females) who had completed their growth. Among them, one exceptionally tall woman with Marfan syndrome and two patients who had already reached his/her final height at the time of diagnosis (M=1 and F=1) were excluded in the analysis of treatment outcomes. In this group of 25 patients (M=17, F=8), the mean follow-up duration was 9.5 ± 6.5 years. After hormone replacement therapy (HRT), IHH patients achieved full development of secondary sex characteristics and appeared as normal adults. Males with nIHH and those with KS did not differ significantly in any clinical parameters, so their data were pooled for analysis. Likewise, the clinical characteristics of females with KS were similar to those of females with nIHH, and their data were also pooled. Table 6 shows the height outcomes following HRT. The average age at the start of hormone therapy was 15.5 ± 0.5 years for males with IHH and 15.7 ± 0.5 years for females with IHH (*p* value=0.53). At that time, the bone age was 14.2 ± 0.3 years for males and 13.1 ± 0.4 years for females (*P* value=0.01). Males with IHH had a height standard deviation score (SDS) of -1.1 ± 0.2 at the beginning of HRT, and their final height SDS was 0.1 ± 0.2, which was significantly increased by 1.2 ± 0.4. Females with IHH had a height SDS of -0.3 ± 0.4 at the start of HRT and reached a final height SDS of 0.7 ± 0.4. Both male and female patients demonstrated a statistically significant increase in height following hormone replacement therapy (HRT), as indicated by a *p* value of less than 0.05. There were no statistically significant differences between males and females in terms of their final height and target height. Besides, it was found that one male patient underwent

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successful in vitro fertilization (IVF) after undergoing gonadotropin ovulatory stimulation, while another male patient preserved sperm successfully following human chorionic gonadotropin (hCG) therapy.

Comparison of IHH patients with versus without a genetic etiology

We categorized the patients based on their genetic etiology into two groups: a genetically resolved group (GR), and a genetically unresolved group (GUR) which including variants of VUS groups and digenic groups. However, we did not observe any significant differences between these groups in terms of diagnostic age, hormone data, or height outcomes (Table 7). Furthermore, no statistically significant difference was observed in the interval of hormone therapy between male patients in the genetically resolved group and those in the genetically unresolved group. This finding suggests that the resolution of genetic factors does not have a significant impact on the frequency of hormone therapy in this population.

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1.4 Discussion

Clinical characteristics and hormone data of IHH patients

This investigation involved 33 patients from Taiwan with IHH, and our findings revealed a higher prevalence of males (n=21) than females (n=12), which aligns with prior research[23,24]. This gender disproportion may be attributable to genes that follow X-linked recessive inheritance patterns, such as *ANOS1* and *SOX3.* Additionally, *FGFR1*, which is another causative gene, may exhibit incomplete penetrance in some families with a higher incidence of affected males[16]. In males, IHH often presents with micropenis and cryptorchidism during infancy, followed by delayed puberty or gynecomastia during adolescence. Conversely, female patients usually experience delayed puberty, arrested puberty, or amenorrhea. As a result, males are typically diagnosed earlier than females, which concurs with previous investigations[25,26]. This trend is particularly noticeable in patients with *ANOS1* variants that are known to cause IHH.

Exome sequencing for targeted IHH-associated genes with 60 % detection rate

Our study involved analyzing a panel of 283 genes associated with IHH. Among the 33 patients included in the study, 18 were found to have P/LP variants in known genes related to hypothalamic and pituitary gland development, GnRH or olfactory neuron development, migration, and regulation. Further investigation revealed two more patients with pathological variants in the *SOX11* gene. The overall detection rate of P/LP variants in IHH patients was 60% which is higher to other reports[4,27]. These results underscore the potential contribution of our gene panel as an effective screening tool to enhance diagnostic efficiency. In cases where patients do not exhibit any specific gene findings within the panel, a thorough review of WES data is conducted to ensure the

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identification of possible missing variants in these individuals. This approach aims to further augment the diagnostic accuracy and comprehensive evaluation of patients, particularly those presenting with atypical genetic profiles.

Pathogenic variants of *FGFR1, CHD7, ANOS1, HS6ST1, PROKR2* and *FGF8*

In our study, *FGFR1*, *CHD7*, and *ANOS1* were identified as the most frequently occurring causative genes, with frequencies of 30%, 25%, and 20%, respectively (Table 4). *FGFR1*, a receptor tyrosine kinase found on chromosome 8q11.2, is activated by anosmin-1, a protein encoded by the *ANOS1* gene on the X chromosome. This activation induces neurite outgrowth and cytoskeletal rearrangement in human embryonic GnRH olfactory neuroblasts[28]. While variants of the *ANOS1* gene are always associated with KS, variants of *FGFR1* can result in KS or nIHH. In cases of IHH patients with anosmia and other syndromes of renal agenesis and synkinesia, as well as a positive maternal family history, a pathogenic variant of the *ANOS1* gene should be considered. *FGFR1* may be associated with limb anomalies, cleft palate, or cleft lip. In our investigation, we observed three patients who possessed a truncating variant in *ANOS1*. Among them, two patients (Patient 12 and 14) had a maternal uncle with infertility and anosmia, indicating a possible X-linked inheritance pattern, which led to an early diagnosis. Patient 13 was born with micropenis, cryptorchidism, and left kidney hypoplasia. A GnRH test was performed during minipuberty suspicion of Kallmann syndrome, which was confirmed. Among the patients with *FGFR1* variants, four were male, and three had pathogenic frameshift variants. Patient 2, who had a frameshift *FGFR1* variant, had split hands and foot malformation. Patient 3 had a missense variant in *FGFR1* (p.Trp666Gly), which was considered pathogenic, as reported in a KS patient with a cleft palate who had another amino acid substitution in the same position (p.Trp666Arg)[28]. Patient 4 had KS and an *FGFR1* variant (p.Ile538Met), and a previous

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report showed another amino acid change at the same position (p.Ile538Val) in a KS family. I538 is in the FGFR kinase domain, and the hydrophobic interaction between I538 and F642 is crucial for ATP binding[31]. Furthermore, Case 6 was identified through genetic testing after her older sister was diagnosed with IHH. Subsequently, it was found that she had the same variant of *FGFR1* (p.Tyr99Ter) as her mother, older sister, and twin brother, as illustrated in Figure 2.

CHD7, a chromodomain-helicase-DNA-binding protein, is expressed in the olfactory placode and plays a crucial role in the development of GnRH neurons, the spinal cord, and other organs. Pathogenic variants in *CHD7* have been associated with a wide spectrum of phenotypes, ranging from CHARGE syndrome to isolated hypogonadotropic hypogonadism (IHH). Our study included five patients with *CHD7* variants, of whom one (Case 7) had incomplete CHARGE syndrome with KS, three had KS, and two had nIHH. Although previous studies have suggested that variants in *CHD7* are more commonly associated with KS rather than nIHH, recent reports have shown that both KS and nIHH can occur in patients with pathogenic *CHD7* variants. Truncating variants in *CHD7* were identified as pathogenic variants in patients 7 and 9, while patients 8 and 10 had missense variants (p.Thr1027Pro, p.Tyr1075Cys) located in the functional domain of SNF2, which are believed to affect protein function, and were therefore classified as likely pathogenic variants[30].

Both FGFR1 and anosmin-1 require Heparan-sulfate proteoglycans (HSPGs) as co-receptors for FGFR1 signaling. Heparan-sulfate 6-O-sulfotransferase 1 (HS6ST1) plays a critical role in this process[28]. In our investigation, Patient 18 exhibited KS without any other IHH-associated gene variants. This was due to an *HS6ST1* likely pathogenic variant (p.Thr203Met) that was considered likely pathogenic based on its severity and

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phenotype. Prokineticin receptor-2 (PROKR2) is involved in the GnRH signaling pathway[31]. Patient 13 exhibited nIHH and had a *PROKR2* missense variant (p.Arg85His) previously reported in a KS patient. Family studies of *PROKR2* have shown a variable phenotype ranging from unaffected individuals to KS with the same pathogenic variant. This suggests that additional genetic or non-genetic factors are involved in the development of the disease. Fibroblast growth factor 8 (FGF8) plays a role in GnRH neuron development and is intimately tied to the development of the olfactory epithelium and/or bulb[32]. Patient 20 was found to have a pathogenic variant in *FGF8* (p.Gln23Ter), which resulted in a truncation of the protein. On the other hand, Patient 5 initially presented with a digenic variant involving both *FGFR1* (p.Thr187HisfsTer5) and *FGF8*(p.Thr229Met). Upon further investigation, it was discovered that the *FGF8* variant in question had been inherited from the patient's father. After employing the ORVAL tool, the evidence pointed more strongly to a single dominant variant of *FGFR1*.

***SOX11*: an IHH-associated gene identified by WES analysis and Absent from IHH panel**

In our study, it was found that two patients had pathogenic variants in the *SOX11* gene. Interestingly, both patients exhibited facial dysmorphisms, mental retardation, and normosmic idiopathic hypogonadotropic hypogonadism (nIHH) (Figure 3). One of the patients also had micropenis and cryptorchidism at birth, which was confirmed as IHH through a GnRH test. The *SOX* gene family encodes transcription factors that play a role in sex determination and neuronal development. While *SOX2*, *SOX3*, and *SOX10* have been previously linked to IHH, there have been no reports of IHH caused by a *SOX11* mutation in the literature. In our original IHH gene panel, the *SOX11* gene was not

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included. *SOX11* is a member of the class-C SOX family and is highly expressed in most hypothalamic GnRH neurons in adult mice. Suppression of the SOX11 protein has been shown to decrease GnRH gene expression[33]. Previous reports have linked *SOX11* variants to Coffin-Siris syndrome, a condition characterized by developmental disability, facial dysmorphisms, and clinodactyly of the fifth finger/toe[29]. Notably, two of our nIHH patients exhibited Coffin-Siris syndrome and were found to have de novo *SOX11* mutations (Table 4, Case 16 and 17). Therefore, this is the first report of IHH caused by *SOX11* gene mutations and expands the phenotypes associated with Coffin-Siris syndrome to include hypogonadotropic hypogonadism.

How WGS and RNA-Seq contribute to understanding IHH patients

The technology of WGS enables thorough and systematic genetic testing within integrated health systems, while also facilitating the discovery of underlying causes in both the coding and non-coding regions of the genome[35]. However, the use of genome-wide homozygosity mapping and next-generation sequencing techniques such as gene-targeted sequencing panels, WES and WGS have not been able to identify all mutations impacting on splicing or gene expression. RNA-Seq is a powerful technique that aids in genetic diagnosis by detecting additional mutations, including splicing defects and pathogenic mutations. These mutations, which may not be captured by WES, can cause significant reductions in mRNA gene expression[36]. Also, one study demonstrated that miR-7a2 plays a critical role in normal pituitary development and hypothalamic-pituitary-gonadal function in adulthood, influencing sexual maturation and reproductive function through its effects on pituitary prostaglandin and BMP4 signaling[37]. Thus, we used WGS and RNA-seq to analysis 4 genetically unknown patients. However, we had only identified one patient with an *NF1* variant, which is a missense variant located in

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the exon region and classified as a VUS (Table 5, case 25). The WES analysis suggested the possibility of a filter error, but based on new information, the variant was considered valid since the BAM file was consistent in both WES and WGS analyses (Figure 4).

Understanding Syndromic and Non-Syndromic Diseases: Exploring Phenotype-Genotype Correlations

The results of previous studies indicate that out of 33 patients diagnosed with IHH, three of them had accompanying syndromes, which were considered unrelated to IHH. These syndromes included Marfan syndrome in Case 10 and Alagille syndrome in Case 19. Furthermore, in order to confirm these diagnoses, whole exome sequencing was performed, and the findings are presented in Table 4. Additionally, it has been suggested that the autism spectrum disorder diagnosis in another individual, Case 15, could be linked to previously reported large deletions of *NLGN3* gene[38]. The genetic analysis revealed that two patients, namely Case 16 and Case 17, had *SOX11* variants. It was also noted that Case 17 had the same variant reported previously, and both patients exhibited a similar phenotype characteristic of Coffin-Siris syndrome[39]. Also, the variant identified in Case 7 was found to be the same as in previous reports of CHARGE syndrome (https://www.ncbi.nlm.nih.gov/clinvar/RCV000509342/). Based on the patient's phenotype, it was determined that they exhibited symptoms consistent with incomplete CHARGE syndrome, which included right ear deformity, arthrogryposis, and bilateral sensorineural hearing loss. Based on the genetic discoveries, we presented a classification of phenotype and genotype, as shown in Figure 5.

Outcomes of HRT in the IHH patients

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Compared to girls, boys generally start puberty at a later age, which results in an older bone age at the beginning of hormone replacement therapy (HRT) treatment. Following treatment, both male and female patients achieved their full height potential, and there was a significant improvement in their secondary sexual characteristics. Notably, there were no discernible differences in clinical presentation, hormonal data, or height outcomes based on identification of the genetic etiology.

Despite the potential infertility complications associated with IHH, one of our patients were able to undergo successful in vitro fertilization (IVF) and had a child due to the availability of advanced reproductive techniques and treatments, which is consistent with previous research[40,41].

Limitations and Future perspectives

Our study was subject to certain limitations. Firstly, we encountered challenges in obtaining the genotypes of some parents for personal reasons, which impeded our ability to confirm the inheritance patterns or classify variants of uncertain significance (VUS) of candidate genes. Secondly, the relatively small size of our study population, owing to the low incidence of IHH, restricted the generalizability of the findings. Nonetheless, we anticipated that the combined use of WGS and RNA-Seq could provide a valuable tool to unravel more complex and comprehensive functional genotype-phenotype associations, beyond those based solely on Mendelian inheritance patterns.

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1.5 Conclusion

In summary, this study successfully identified the genetic causes of most cases (60 %) of IHH, with a higher prevalence observed among male patients. Exome sequencing coupled with targeted analysis of IHH-related genes proved to be an effective method for identifying both known and unknown genes responsible for the condition. A total of 20 pathogenic or likely pathogenic variants were identified, with the newly discovered *SOX11* gene being implicated as a causative gene for IHH. This study provides a comprehensive analysis of the clinical, hormonal, genetic, and treatment data of Taiwanese patients with IHH. Verification of these candidate genes has the potential to not only guide treatment plans but also advance the understanding of GnRH neuronal migration and HPG axis. More cases are required to furnish supplementary proof for the efficiency of RNA-seq and WGS in patients with IHH.

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Chapter 2. Tables and Figures

2.1 Tables

Table 1. IHH panel including 283 genes

ACSM4	*FAR2*	*HCRT*	*NRP2*	*SCGB1c1*
AEBP2	*FEZF1*	*HCRTR1*	*NSCL2*	*SCIP*
AKAP1	*FGF1*	*HESX1*	*NSMCE4A*	*SDR16C5*
AKAP2	*FGF10*	*HGF*	*NELF*	*SEC14L3*
AKAP3	*FGF11*	*HS6ST1*	*NTN*	*SEC23IP*
AKAP4	*FGF12*	*IFT57*	*NTN1*	*SEMA3A*
ALDH1A1	*FGF13*	*IGF1*	*OBP2B*	*SEMA3E*
ALDH1A2	*FGF14*	*IGF1R*	*OCT1*	*SEMA4D*
AMN1	*FGF16*	*IGSF10*	*OMP*	*SEMA7A*
ANKRD26	*FGF17*	*IL17RD*	*OTUD4*	*SFRP1*
ARNTL2	*FGF18*	*IPO8*	*OTX2*	*SFRP5*
ASUN	*FGF19*	*JAG1*	*OVCH1*	*SH2D3C*
ATE1	*FGF2*	*KAL1*	*P75NTR*	*SHH*
AXL	*FGF20*	*KCNK9*	*PAX6*	*SIX3*
BDNF	*FGF21*	*KCTD11*	*PAX7*	*SIX6*
BMP2	*FGF22*	*KIAA0528*	*PCDH8*	*SLC17A6*
BMP4	*FGF3*	*KISS1*	*PCSK1*	*SLCO1C1*
BMP7	*FGF4*	*KISS1R*	*PDE3A*	*SMAD3*
BPIFB4	*FGF5*	*KLB*	*PENK*	*SMAD4*
BRN2	*FGF6*	*KLF6*	*PIT1*	*SMAD5*
C12ORF70	*FGF7*	*KLHDC5*	*PITX2*	*SMCHD1*
C12ORF71	*FGF8*	*LEP*	*PKNOX1*	*SMO*
CACNA1B	*FGF9*	*LEPR*	*PKNOX2*	*SOX2*
CADM2	*FGFR1*	*LHB*	*PLEKHA5*	*SOX3*
CAPRIN2	*FGFR1OP2*	*LHX3*	*PLXNA1*	*SP8*
CAS	*FGFR2*	*LHX4*	*PLXNB1*	*SPRY4*
CCDC141	*FGFR3*	*LHX5*	*PNPLA6*	*SRA1*
CCDC91	*FLRT3*	*LIM1*	*POLR3A*	*ST8SIA1*
CCKAR	*FOXH1*	*LRP8*	*POLR3B*	*STIL*
CCKBR	*FSHB*	*MANSC4*	*POMC*	*STK38L*
CGA	*FSTL5*	*MAP1L*	*POU1F1*	*STMN1*
CHAT	*GABABR1*	*MAPK14*	*POU5F1*	*STUB1*
CHD7	*GADL1*	*MASH1*	*PPAPDC1A*	*SUFU*

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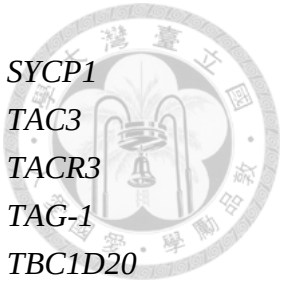

CMAS	*GAL*	*MASTL*	*PPFIBP1*	*SYCP1*
CNTN2	*GALR1*	*MATH4A*	*PREP1*	*TAC3*
CRY1	*GALR2*	*MCMBP*	*PROK2*	*TACR3*
CRY2	*GALR3*	*MED21*	*PROKR1*	*TAG-1*
CTXN3	*GAP43*	*MEIS1*	*PROKR2*	*TBC1D20*
CXCL12	*GAS6*	*MEOX1*	*PROP1*	*TBR1*
CXCR4	*GATA2*	*MET*	*PTC1*	*TBX2*
DCC	*GATA3*	*METTL20*	*PTCH1*	*TGFB*
DHH	*GATA4*	*MRPS35*	*PTCH2*	*TM7SF3*
DLX1	*GDF1*	*MSX1*	*PTHLH*	*TMTC1*
DLX2	*GDNF*	*MSX2*	*PTX1*	*TRAPPC9*
DLX5	*GLI1*	*MYCN*	*RAB18*	*TRIM25*
DMXL2	*GLI2*	*NCAM*	*RAB3GAP1*	*TSPAN11*
DUSP6	*GLI3*	*NDN*	*RAB3GAP2*	*TUBB2A*
EBF2	*GNRH1*	*NEUROD*	*RAC*	*TUBB2B*
EFNA5	*GNRHR*	*NEUROG2*	*RD3*	*TUBB3*
EGFR	*GPX6*	*NF1*	*RELN*	*UMODL1*
EGR1	*GRG*	*NK2*	*REP15*	*WDR11*
EMX1	*GRG4*	*NODAL*	*RNF216*	*WNT11*
EMX2	*GRG5*	*NOS1*	*RTP1*	*WNT8b*
ENPP1	*GRIN1*	*NOTCH1*	*RTP2*	*YME1L1*
ERGIC2	*GRIN2*	*NR0B1*	*RUNX1*	*ZFP423*
ETNK1	*GSTM1*	*NR5A1*	*RUNX2*	
FAM60A	*H3F3C*	*NRP1*	*RUNX3*	

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Table 2. Clinical features of IHH

Phenotype	nIHH (n=17)		Kallmann syndrome (n=16)		*P* value
Patient's number	**Male (n=8)**	**Female (n=9)**	**Male (n=13)**	**Female (n=3)**	
Endocrine					
Micropenis	6	-	10	-	0.92
Crytorchidism	2	-	6	-	0.33
No spontaneous puberty	6	5	11	2	0.23
Arrested puberty	1	4	1	1	0.27
Gynecomastia	2	-	1	-	0.67
Amenorrhea	-	9	-	3	1.00
Non-Endocrine					
Hearing loss	1	0	0	1	0.97
Facial dysmorphism	3	0	0	1	0.32
Renal dysgenesis	0	0	1	0	0.30
Bony defects	3	0	0	1	0.32
Mental retardation	2	0	1	0	0.58

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Table 3. Hormone data by phenotype

	nIHH(n=17)		KS(n=16)		*P* value
	Male(n=8)	Female(n=9)	Male(n=13)	Female(n=3)	
Age at diagnosis (years)	13.3 ±5.0	15.6 ± 1.9	12.2±6.9	19.2 ± 5.3	0.92
Bone age at diagnosis (years)*	14.3 ± 1.8	13.2 ± 1.3	14.1± 0.8	12.5± 0.0	0.34
Baseline					
FSH (mIU/mL)	1.2 ± 1.2	0.4 ± 0.3	0.7 ± 0.7	0.5 ± 0.1	0.72
LH (mIU/mL)	0.6 ± 0.9	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.34
Testosterone (ng/mL)	0.3 ± 0.2	-	0.2 ± 0.0	-	0.87
Estradiol (pg/mL)	-	20.4 ± 5.6	-	24.5 ± 7.4	0.15
GnRH test					
Peak FSH (mIU/mL)	5.5 ± 3.0	4.2 ± 2.7	5.1 ± 3.5	3.7 ± 1.9	0.80
Peak LH (mIU/mL)	7.1 ± 5.6	5.1 ± 5.7	5.1 ± 4.8	2.6 ± 0.6	0.63

FSH: follicle-stimulating hormone, GnRH: gonadotropin-releasing hormone, KS: Kallmann syndrome, LH: luteinizing hormone, nIHH: normosmic idiopathic hypogonadotropic hypogonadism

* Two male patients with KS and a male patient with IHH were not included in bone age assessment due to being too young (below 2 years old). A female patient with KS, who had a fused bone age, was also excluded.

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Table 4. Phenotype and Genotype Classification into Pathogenic/Likely Pathogenic Groups

Gene/NM number	Case	Age at diagnosis/Sex	Phenotype	Nucleotide change	Amino acid change	Zygosity/ Inheritance	Classification (ACMG)/Score*
FGFR1/ 023110.3	1	14/M	KS	c.1037_1038delCT	p.Ser346TyrfsTer61	Het, AD	P (PVS1,PP5,PM2)/17
	2	14/M	nIHH[a]	c.1038 dupT	p.Ile347TyrfsTer61	Het, AD	P (PVS1,PM2,PS1)/13
	3	15/M	nIHH	c.1996T>G	p.Trp666Gly	Het, AD	P (PM5,PP3,PM1,PM2)/11
	4	14/M	KS	c.1614C>G	p.Ile538Met	Het[h], AD	LP(PM1,PM2,PM5,PP2,PP5,BP4)/6
	5	16/F	KS	c.558delC	p.Thr187HisfsTer5	Het, AD	P (PVS1, PM2, PS2)/13
	6	14/F	nIHH	c.297T>G	p.Tyr99Ter	Het[i], AD	P (PP4,PVS1,PM2)/10
CHD7/ 017780.4	7	16/F	KS[b]	c.3655C>T	p.Arg1219Ter	Het, AD	P (PVS1,PP3, PP5,PM2)/18
	8	17/M	KS	c.3079A>C	p.Thr1027Pro	Het, AD	LP (PM1,PM2,PM6,PP3)/9
	9	13/M	nIHH	c.8137_8138delGG	p.Gly2713Serfs Ter11	Het, AD	P(PVS1,PM2,PM6)/11
	10	17/F	nIHH[c]	c.3224A>G	p.Tyr1075Cys	Het, AD	LP (PM1,PM2,PM6,PP3,PP4)/7
	11	25/F	KS	c.2656C>T	p.Arg886Trp	Het, AD	LP (PM2,PP3,PP4,PP5,BP1)/6

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ANOS1/ 000216.4	12	12/M	KS	c.1097delC	p.Pro366GlnfsTer12	Hemi[i], XL	P (PVS1,PM2,PP4)/11
	13	0.3/M	KS[d]	c.1267C>T	p.Arg423Ter	Hemi[i], XL	P(PVS1,PM2,PP5,PS1)/17
	14	1/M	KS	c.784C>T	p.Arg262Met	Hemi[i], XL	P(PVS1,PM2,PP5)/13
	15	17/M	KS[e]	6628264_8797517 del(2.17Mb)	-	Hemi, XL	P(PVS1,PM2,PS1)/13
SOX11/ 003108.4	16	14/M	nIHH[f]	c.325C>A	p.His109Asn	Het, AD	P (PS2,PM1,PM2,PP3,PP4)/12
	17	2/M	nIHH[f]	c.347A>G	p.Tyr116Cys	Het, AD	P(PS1,PS2,PM1,PM2,PP3,PP5)/17
HS6ST1/ 004807.3	18	15/M	KS	c.608C>T	p.Thr203Met	Het, AD	LP(PM1,PM2,PP3,PP5,BP1)/6
PROKR2/ 144773.4	19	20/M	nIHH[g]	c.254G>A	p.Arg85His	Het[h], AD	P(PS1,PS3,PM2,PM5,PP5,BP4)/14
FGF8/ 033163.5	20	19/M	KS	c.67C>T	p.Gln23Ter	Het, AD	P(PVS1,PM2,PM6)/11

ACMG: American College of Medical Genetics Laboratory Practice Committee Working Group, AD: autosomal dominant, Hemi: hemizygous. Het: heterozygous, KS: Kallmann syndrome, nIHH: normosmic idiopathic hypogonadotropic hypogonadism, PM: pathogenic moderate, PP: pathogenic supporting, PS: pathogenic strong, PVS: pathogenic very strong, XL: X-linked recessive.

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A: digital bone malformation, b: hearing loss, ear deformity, facial dysmorphism, incomplete CHARGE syndrome, c: Marfan syndrome: NM_000138.4(*FBN1*): c.3455C > T (p.Ala1152Val), d: renal agenesis, e: Autism spectrum disorder and attention-deficit hyperactivity disorder, NM_001166660.2(*NLGN3*): 6628264_8797517 del (2.17Mb), f: facial dysmorphism, mental retardation, g: Alagille syndrome: NM_000214.3 (*JAG1*): c.689_690insTGTAA (p.R231Vfs*182), Rathke cleft cyst, s/p surgical removal h: inherited from father. I: inherited from mother.

*Points: Rules are combined using the point system described in PMID:32720330. The total score is compared to thresholds to assign the final verdict: pathogenic：≥10, likely pathogenic: 6-9 inclusive, uncertain significance: 0-5.

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Table 5. Phenotype and Genotype Classification into Variants of Uncertain Significance/Digenic Variants Groups

Case	Age at diagnosis/ Sex	Phenotype	Gene/ NM_number	Nucleotide change/ Amino Acid change	Zygosity /Digenic/ Inheritance	Classification (ACMG)/ Score	ORVEL median pathogenicity score
21	14/M	KS	*FSTL5*/ 00128428.3	c.G2390C p.Gly797Ala	Het	-	0.81
			NOS1/ 000620.5	c.A1889T p.Asp630Val	Het	-	
22	14/F	nIHH	*DCC*/ 005215.4	c.A4324G p.Ile1442Val	Het	-	0.68
			PNPLA6/ 006702.5	c.A3100G p.Thr1034Ala	Het	-	
23	17/M	KS[a]	*EBF2*/ 022659.4	c.G1471A p.Gly491Ser	Het	-	0.70
			PDE3A/ 000921.5	c.A82G p.Met28Val	Het	-	

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24	18/F	nIHH	*NOTCH1* /017617.5	c.G751A p.Gly251Ser	Het[b], AD	VUS(PP3,PM 2,BP1)/4	-
25	15/F	nIHH	*NF1/* 00104292.3	c.G3749A p.Arg1250Gln	Het, AD	VUS(PM5,PM 1,PM2,PP3)/5	-

AD: autosomal dominant, Het: heterozygous KS: Kallmann syndrome, nIHH: normosmic idiopathic hypogonadotropic hypogonadism

a: mental retardation b. Inherited from father

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Table 6. Height Outcomes after HRT in patients with IHH

	Male (n=17)	Female (n=8)	*P* value
Age at diagnosis (years)	13.2± 1.2	16.5 ± 0.9	0.09
Age at start of treatment (years)	15.5 ± 0.5	15.7 ± 0.5	0.53
Bone age at start of treatment (years)	14.2 ± 0.3	13.1 ± 0.4	0.01
Target height SDS	0.0 ± 0.2	-0.3 ± 0.3	0.48
Height SDS at start of treatment	-1.1 ± 0.2	-0.3 ± 0.4	0.14
Final height SDS	0.1 ± 0.2	0.7 ± 0.4	0.16*

SDS: standard deviation score

*****Using Wilcoxon sign rank test to compare before and after HRT treatment, males *P* value: 0.0000, females *P* value: 0.0078

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Table 7. Data Comparison between Genetically Resolved group (GR) and Genetically unresolved group (GUR)

	GR(n=20)	GUR (n=13)	P value
Age at diagnosis(years)	13.6± 6.3	14.2±4.5	0.85
Chronological age(years)	15.1± 2.1	15.0±1.8	0.93
Bone age at diagnosis(years)	13.9± 1.5	13.8±0.9	0.60
Baseline			
FSH (mIU/mL)	0.9± 1.0	0.5±0.4	0.06
LH (mIU/mL)	0.4± 0.6	0.2±0.1	0.20
GnRH test			
Peak FSH (mIU/mL)	5.1±3.3	4.4±2.6	0.46
Peak LH (mIU/mL)	5.7±5.2	4.9±4.9	0.99
Height SDS at start of treatment	-0.7±0.4	-0.6±0.3	0.75
Final height SDS	0.6±0.3	0.3±0.3	0.69

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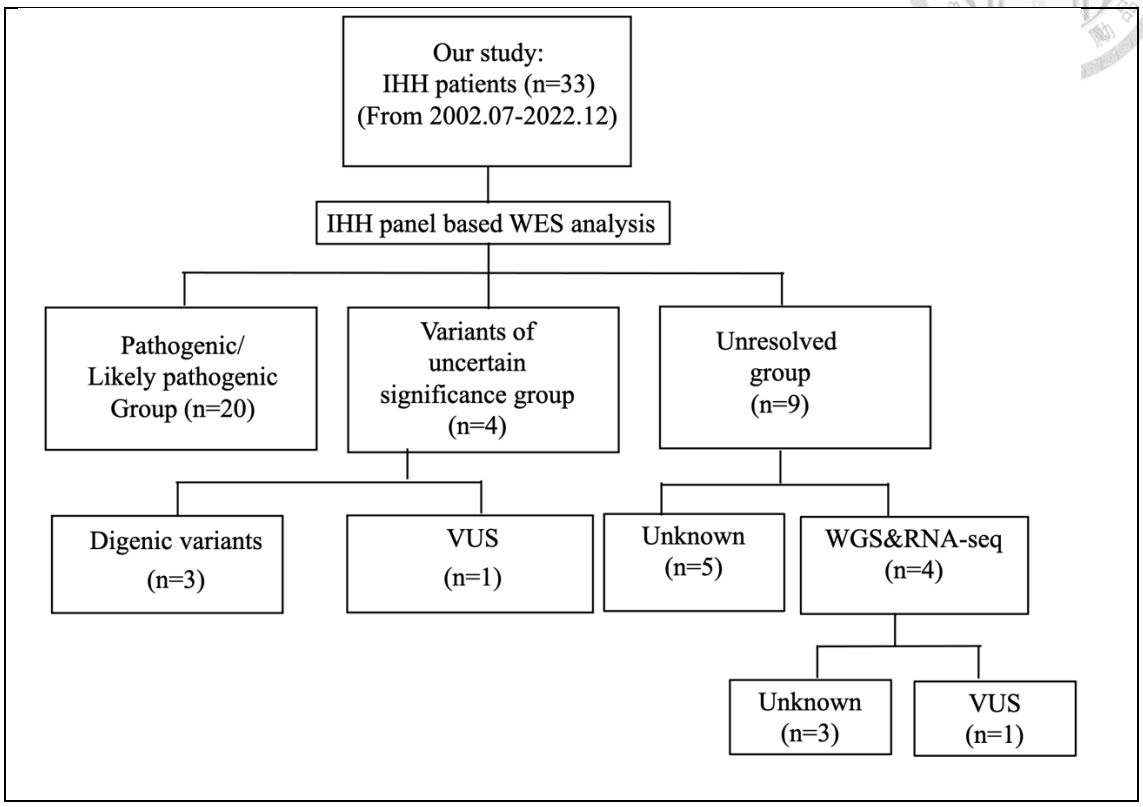
2.2 Figures

Figure 1. Study Flowchart



IHH: idiopathic hypogonadotropic hypogonadism, RNA-seq: RNA sequencing, VUS: variants of uncertain significance, WES: whole exome sequencing, WGS: whole genome sequencing

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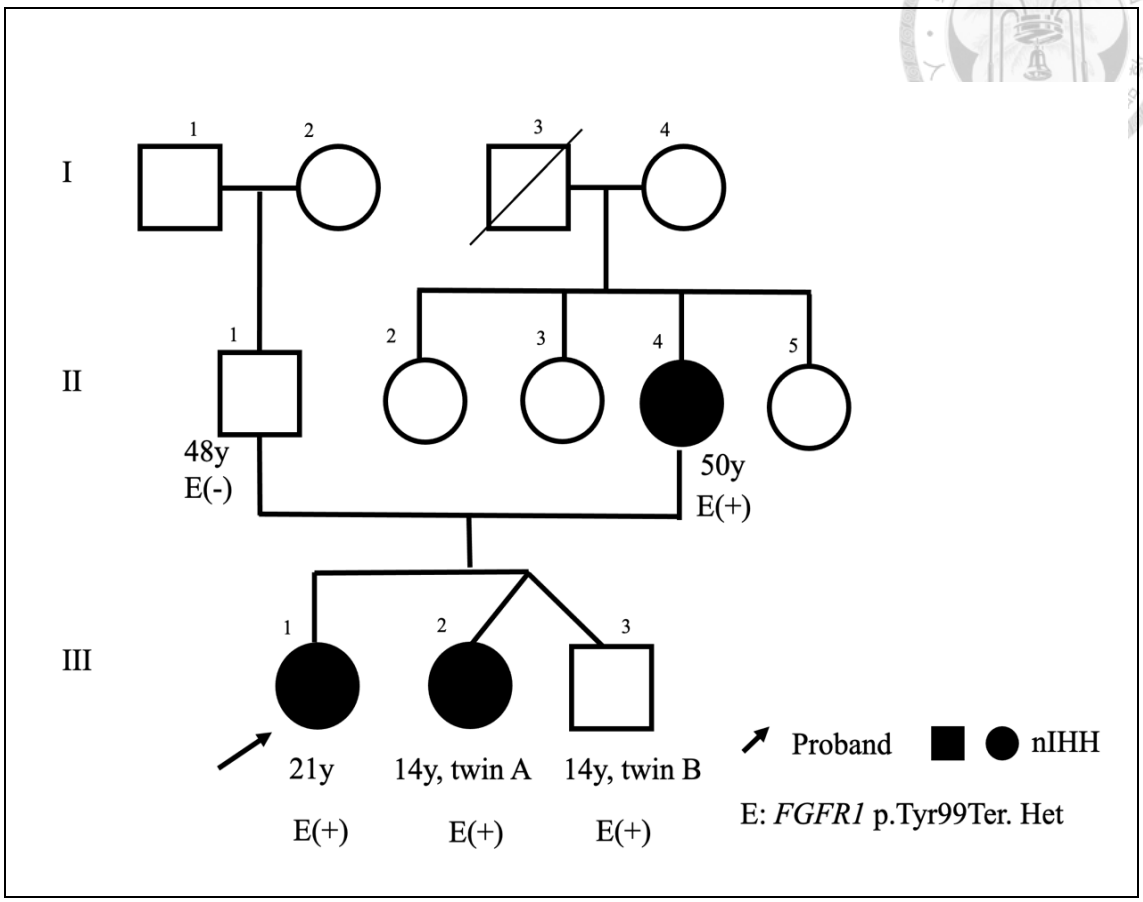
Figure 2. An example of *FGFR1* Variant in One Family



A benchmark case (proband) presented to our hospital with amenorrhea and was subsequently diagnosed with hypogonadotropic hypogonadism. During the investigation, it was discovered that the proband's mother had undergone in vitro fertilization (IVF) procedures twice during her youth. To further elucidate the genetic basis of the condition, the family underwent whole exome sequencing (WES) which confirmed the presence of an *FGFR1* variant, but not in her father.

E: an evaluation of clinical survey, including gene test, nIHH: normosmic idiopathic hypogonadotropic hypogonadism

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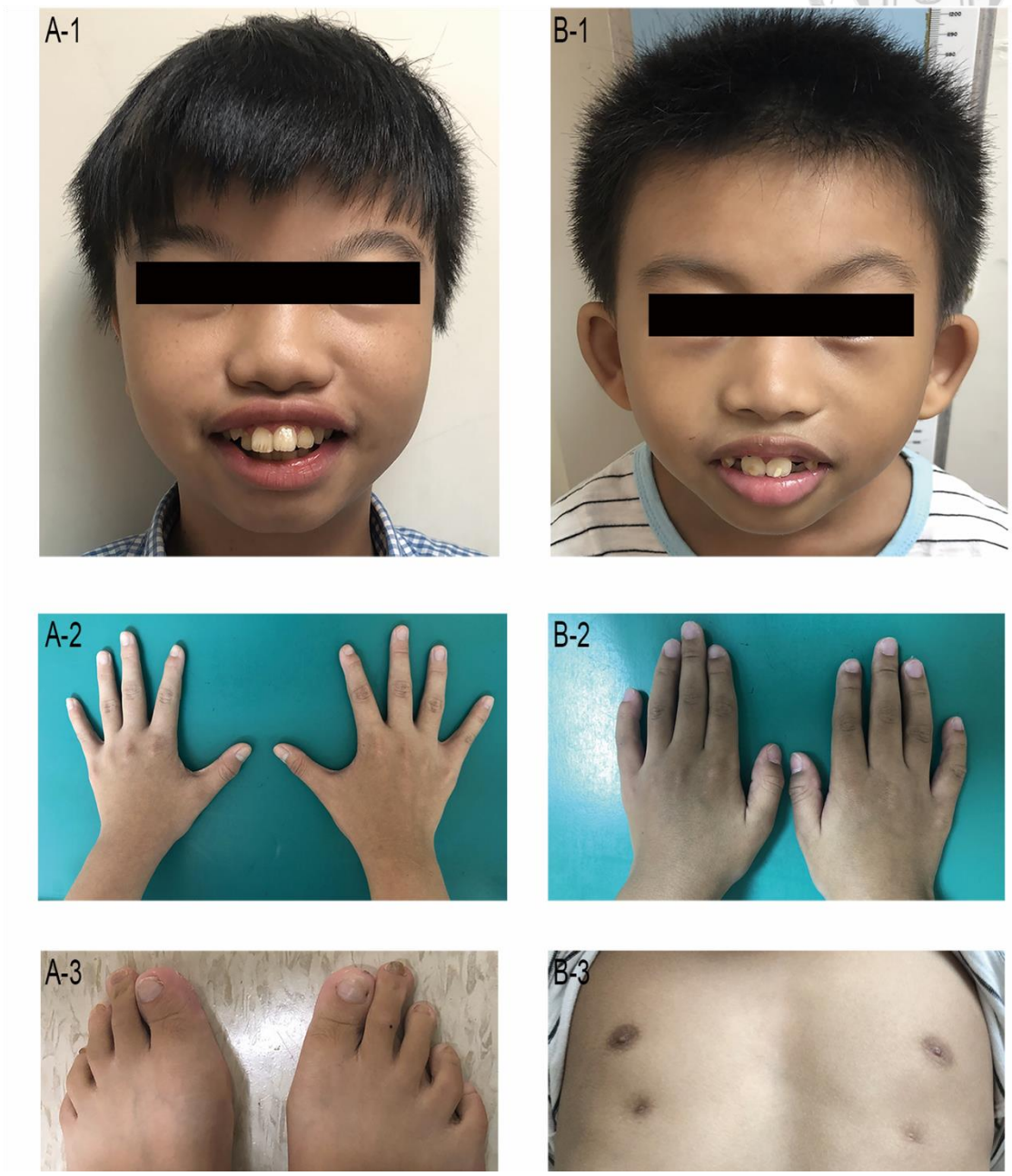
Figure 3. Phenotypes of the patients with SOX11 mutations



(A) Case 16: Facial dysmorphism was observed in a 16-year-old male with IHH, characterized by a brief philtrum, epicanthal folds, hypertelorism, noticeable incisors, and misaligned teeth (A-1). In addition, he experienced mild intellectual disability and delayed speech. The presence of fifth finger clinodactyly (A-2) and small nails on the fourth and fifth fingers (A-3) were also observed. Further analysis confirmed the

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presence of a heterozygous p.His109Asn mutation in the *SOX11* gene. (B) Case 17: Facial dysmorphism, malalignment of teeth, and low-set ears were observed in a 7-year-old male (B-1), accompanied by intellectual disability (IQ of 61). Clinodactyly of the fifth fingers (B-2) and the presence of supranumerary nipples (B-3) were also noted. Since birth, the patient had micropenis with cryptorchidism, and a GnRH test showed IHH during the minipuberty period. Further analysis confirmed the presence of a heterozygous p.Tyr116Cys mutation in the *SOX11* gene.

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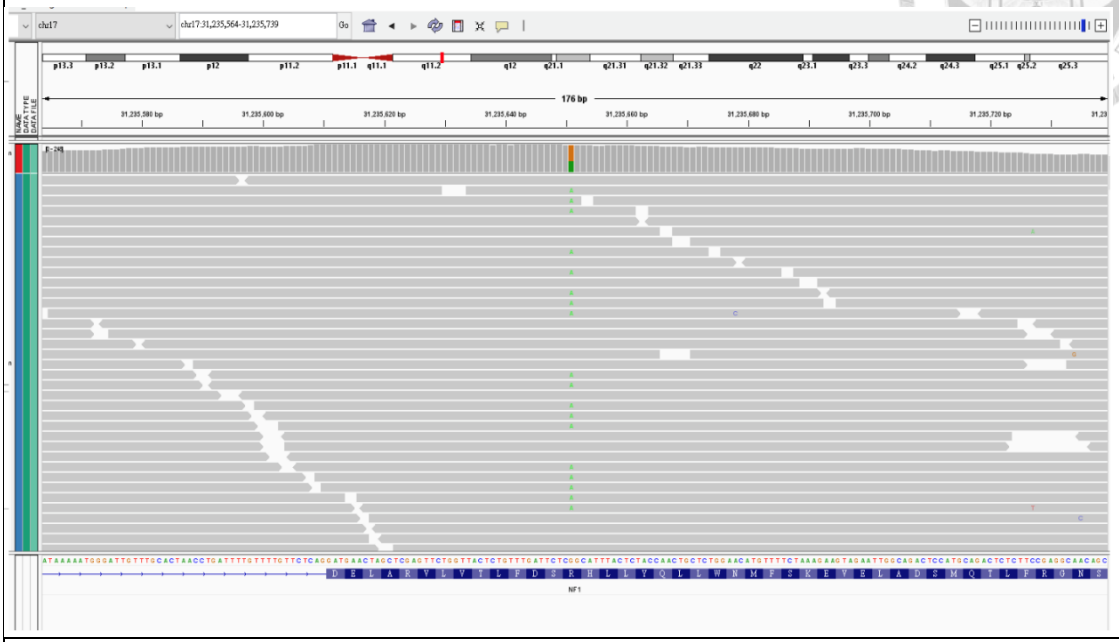
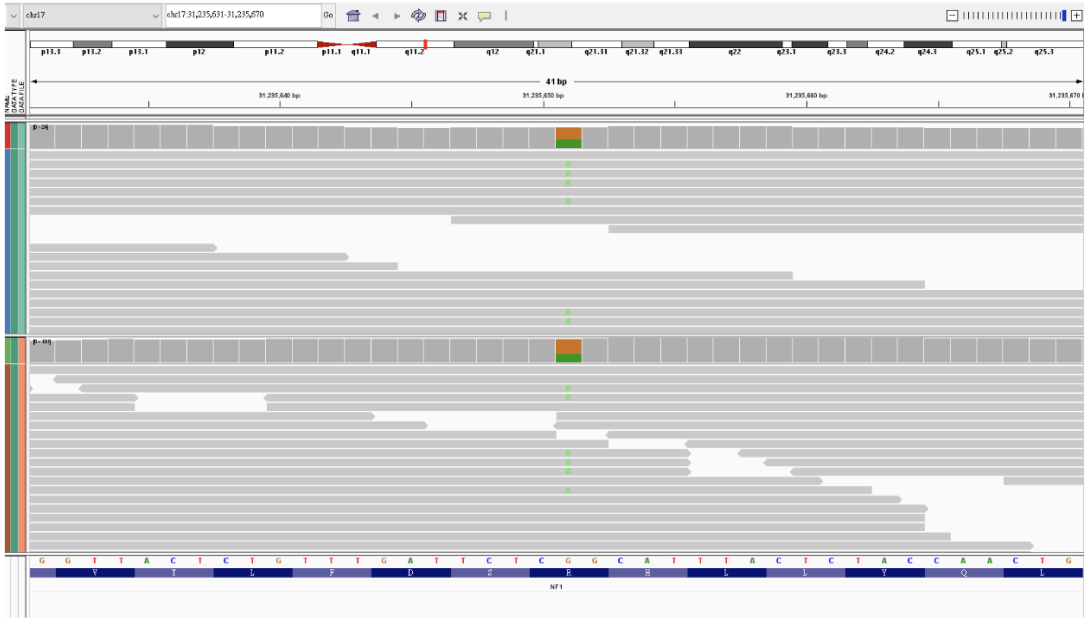
Figure 4. *NF1* variant in WES, WGS and RNA-seq

4a



4b



5a. BAM file in WES analysis 5b. BAM files in WGS and RNA-seq analyses

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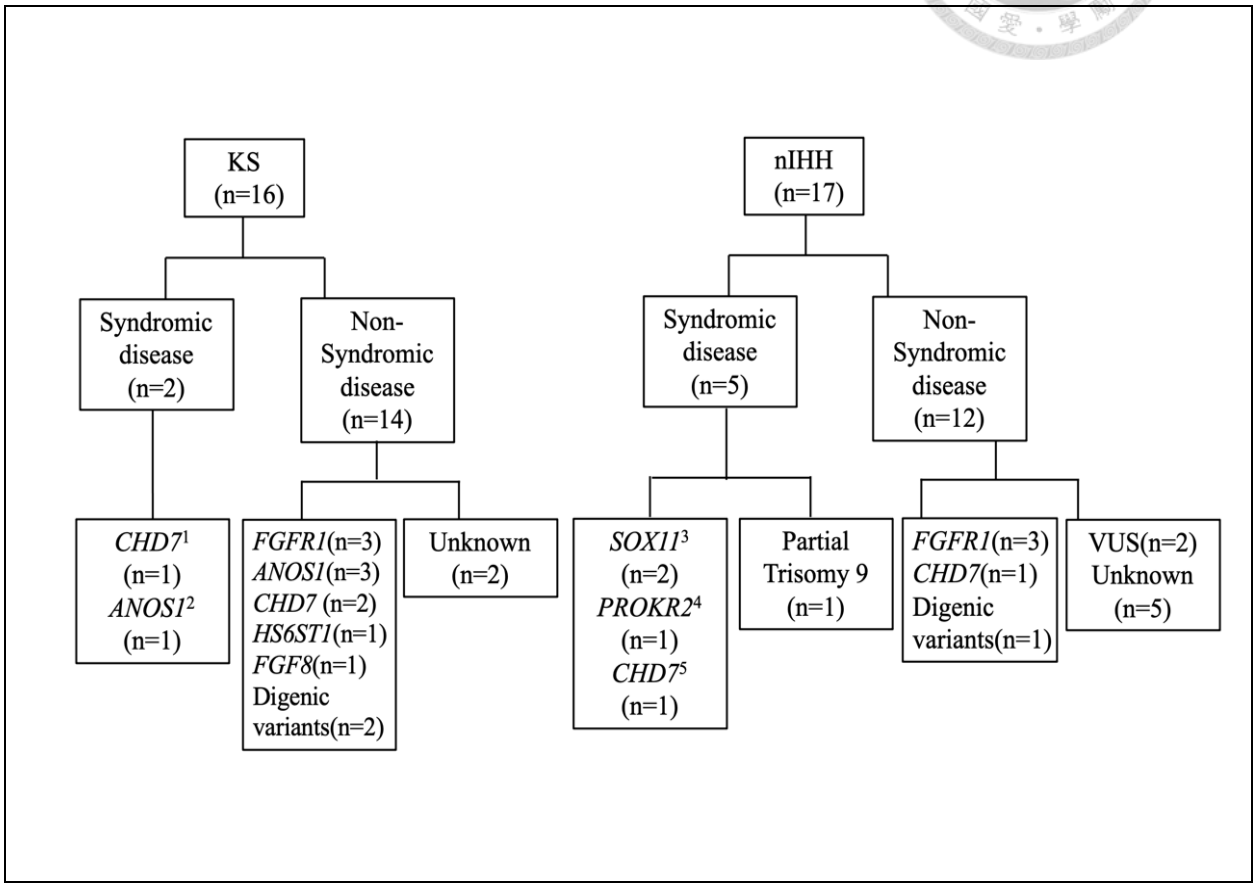
Figure 5. Phenotype-Genotype Correlations in Syndromic and Non-Syndromic Diseases



Other phenotype and disease: 1: incomplete CHARGE syndrome 2: Autism spectrum disorder and attention-deficit hyperactivity disorder 3: Coffin-Siris like syndrome 4: Alagille syndrome 5: Marfan syndrome

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參考文獻 References

1. Bianco SD, Kaiser UB. The genetic and molecular basis of idiopathic hypogonadotropic hypogonadism. Nat Rev Endocrinol. 2009;5(10):569-576. doi:10.1038/nrendo.2009.177

2. Cho HJ, Shan Y, Whittington NC, Wray S. Nasal Placode Development, GnRH Neuronal Migration and Kallmann Syndrome. Front Cell Dev Biol. 2019;7:121. Published 2019 Jul 11. doi:10.3389/fcell.2019.00121

3. Pitteloud N, Quinton R, Pearce S, et al. Digenic mutations account for variable phenotypes in idiopathic hypogonadotropic hypogonadism. J Clin Invest. 2007;117(2):457-463. doi:10.1172/JCI29884

4. Zhou C, Niu Y, Xu H, et al. Mutation profiles and clinical characteristics of Chinese males with isolated hypogonadotropic hypogonadism. Fertil Steril. 2018;110(3):486-495.e5. doi:10.1016/j.fertnstert.2018.04.010

5. Maione L, Dwyer AA, Francou B, et al. Genetic counseling for congenital hypogonadotropic hypogonadism and Kallmann syndrome: new challenges in the era of oligogenism and next-generation sequencing. Eur J Endocrinol. 2018;178(3):R55-R80. doi:10.1530/EJE-17-0749

6. Cassatella D, Howard SR, Acierno JS, et al. Congenital hypogonadotropic hypogonadism and constitutional delay of growth and puberty have distinct genetic architectures. Eur J Endocrinol. 2018;178(4):377-388. doi:10.1530/EJE-17-0568

7. Raivio T, Falardeau J, Dwyer A, et al. Reversal of idiopathic hypogonadotropic hypogonadism. N Engl J Med. 2007;357(9):863-873. doi:10.1056/NEJMoa066494

8. Lee HS, Shim YS, Hwang JS. Treatment of congenital hypogonadotropic hypogonadism in male patients. Ann Pediatr Endocrinol Metab. 2022;27(3):176-182. doi:10.6065/apem.2244208.104

doi:10.6342/NTU202301017

9. Young J, Xu C, Papadakis GE, et al. Clinical Management of Congenital Hypogonadotropic Hypogonadism. Endocr Rev. 2019;40(2):669-710. doi:10.1210/er.2018-00116

10. Hsu NI, Lai JT, Shen PH. Development of Taiwan Smell Identification Test: a quick office-based smell screening test for Taiwanese. Am J Rhinol Allergy. 2015;29(2):e50-e54. doi:10.2500/ajra.2015.29.4174

11. Greulich WW, Pyle SI. Radiographic atlas of skeletal development of the hand and wrist. Stanford University Press; 1959.

12. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 2009;25(14):1754-1760. doi:10.1093/bioinformatics/btp324

13. McLaren W, Gil L, Hunt SE, et al. The Ensembl Variant Effect Predictor. Genome Biol. 2016;17(1):122. Published 2016 Jun 6. doi:10.1186/s13059-016-0974-4

14. Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res. 2010;38(16):e164. doi:10.1093/nar/gkq603

15. Dobin A, Davis CA, Schlesinger F, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics. 2013;29(1):15-21. doi:10.1093/bioinformatics/bts635

16. Topaloğlu AK. Update on the Genetics of Idiopathic Hypogonadotropic Hypogonadism. J Clin Res Pediatr Endocrinol. 2017;9(Suppl 2):113-122. doi:10.4274/jcrpe.2017.S010

17. Butz H, Nyírő G, Kurucz PA, Likó I, Patócs A. Molecular genetic diagnostics of hypogonadotropic hypogonadism: from panel design towards result interpretation in clinical practice. Hum Genet. 2021;140(1):113-134. doi:10.1007/s00439-020-02148-0

18. Quaynor SD, Bosley ME, Duckworth CG, et al. Targeted next generation sequencing approach identifies eighteen new candidate genes in normosmic

doi:10.6342/NTU202301017

hypogonadotropic hypogonadism and Kallmann syndrome. Mol Cell Endocrinol. 2016;437:86-96. doi:10.1016/j.mce.2016.08.007

19. Roberts SA, Kaiser UB. Genetic etiologies of central precocious puberty and the role of imprinted genes. Eur J Endocrinol. 2020;183(4):R107-R117. doi:10.1530/EJE-20-0103

20. Renaux A, Papadimitriou S, Versbraegen N, et al. ORVAL: a novel platform for the prediction and exploration of disease-causing oligogenic variant combinations. Nucleic Acids Res. 2019;47(W1):W93-W98. doi:10.1093/nar/gkz437

21. Dodé C, Levilliers J, Dupont JM, et al. Loss-of-function mutations in FGFR1 cause autosomal dominant Kallmann syndrome. Nat Genet. 2003;33(4):463-465. doi:10.1038/ng1122

22. Kingdom R, Wright CF. Incomplete Penetrance and Variable Expressivity: From Clinical Studies to Population Cohorts. Front Genet. 2022;13:920390. Published 2022 Jul 25. doi:10.3389/fgene.2022.920390

23. Mitchell AL, Dwyer A, Pitteloud N, Quinton R. Genetic basis and variable phenotypic expression of Kallmann syndrome: towards a unifying theory. Trends Endocrinol Metab. 2011;22(7):249-258. doi:10.1016/j.tem.2011.03.002

24. Millar AC, Faghfoury H, Bieniek JM. Genetics of hypogonadotropic hypogonadism. Transl Androl Urol. 2021;10(3):1401-1409. doi:10.21037/tau.2020.03.33

25. Wang Y, Gong C, Qin M, Liu Y, Tian Y. Clinical and genetic features of 64 young male paediatric patients with congenital hypogonadotropic hypogonadism. Clin Endocrinol (Oxf). 2017;87(6):757-766. doi:10.1111/cen.13451

26. Boehm U, Bouloux PM, Dattani MT, et al. Expert consensus document: European Consensus Statement on congenital hypogonadotropic hypogonadism--pathogenesis,

doi:10.6342/NTU202301017

diagnosis and treatment. Nat Rev Endocrinol. 2015;11(9):547-564. doi:10.1038/nrendo.2015.112

27. Gach A, Pinkier I, Sałacińska K, et al. Identification of gene variants in a cohort of hypogonadotropic hypogonadism: Diagnostic utility of custom NGS panel and WES in unravelling genetic complexity of the disease. Mol Cell Endocrinol. 2020;517:110968. doi:10.1016/j.mce.2020.110968

28. González-Martínez D, Kim SH, Hu Y, et al. Anosmin-1 modulates fibroblast growth factor receptor 1 signaling in human gonadotropin-releasing hormone olfactory neuroblasts through a heparan sulfate-dependent mechanism. J Neurosci. 2004;24(46):10384-10392. doi:10.1523/JNEUROSCI.3400-04.2004

29. Pitteloud N, Meysing A, Quinton R, et al. Mutations in fibroblast growth factor receptor 1 cause Kallmann syndrome with a wide spectrum of reproductive phenotypes. Mol Cell Endocrinol. 2006;254-255:60-69. doi:10.1016/j.mce.2006.04.021

30. Kim HG, Kurth I, Lan F, et al. Mutations in CHD7, encoding a chromatin-remodeling protein, cause idiopathic hypogonadotropic hypogonadism and Kallmann syndrome. Am J Hum Genet. 2008;83(4):511-519. doi:10.1016/j.ajhg.2008.09.005

31. Dodé C, Teixeira L, Levilliers J, et al. Kallmann syndrome: mutations in the genes encoding prokineticin-2 and prokineticin receptor-2. PLoS Genet. 2006;2(10):e175. doi:10.1371/journal.pgen.0020175

32. Falardeau J, Chung WC, Beenken A, et al. Decreased FGF8 signaling causes deficiency of gonadotropin-releasing hormone in humans and mice. J Clin Invest. 2008;118(8):2822-2831. doi:10.1172/JCI34538

33. Kim HD, Choe HK, Chung S, et al. Class-C SOX transcription factors control GnRH gene expression via the intronic transcriptional enhancer. Mol Endocrinol. 2011;25(7):1184-1196. doi:10.1210/me.2010-0332

doi:10.6342/NTU202301017

34. Hempel A, Pagnamenta AT, Blyth M, et al. Deletions and de novo mutations of SOX11 are associated with a neurodevelopmental disorder with features of Coffin-Siris syndrome. J Med Genet. 2016;53(3):152-162. doi:10.1136/jmedgenet-2015-103393

35. Turro E, Astle WJ, Megy K, et al. Whole-genome sequencing of patients with rare diseases in a national health system. Nature. 2020;583(7814):96-102. doi:10.1038/s41586-020-2434-2

36. Saeidian AH, Youssefian L, Vahidnezhad H, Uitto J. Research Techniques Made Simple: Whole-Transcriptome Sequencing by RNA-Seq for Diagnosis of Monogenic Disorders. J Invest Dermatol. 2020;140(6):1117-1126.e1. doi:10.1016/j.jid.2020.02.032

37. Ahmed K, LaPierre MP, Gasser E, et al. Loss of microRNA-7a2 induces hypogonadotropic hypogonadism and infertility. J Clin Invest. 2017;127(3):1061-1074. doi:10.1172/JCI90031

38. Marco EJ, Skuse DH. Autism-lessons from the X chromosome. Soc Cogn Affect Neurosci. 2006;1(3):183-193. doi:10.1093/scan/nsl028

39. Wakim V, Nair P, Delague V, et al. SOX11-related syndrome: report on a new case and review. Clin Dysmorphol. 2021;30(1):44-49.

40. Prior M, Stewart J, McEleny K, Dwyer AA, Quinton R. Fertility induction in hypogonadotropic hypogonadal men. Clin Endocrinol (Oxf). 2018;89(6):712-718. doi:10.1111/cen.13850

41. Zhang CM, Zhang H, Yang R, et al. The Reproductive Outcome of Women with Hypogonadotropic Hypogonadism in IVF. Front Endocrinol (Lausanne). 2022;13:850126. Published 2022 Jun 6. doi:10.3389/fendo.2022.850126

doi:10.6342/NTU202301017