Clinical profiles and genetic spectra of 814 Chinese children with short stature

Xin Li¹#, Ruen Yao²#, Guoying Chang¹#, Qun Li¹#, Cui Song³, Niu Li², Yu Ding¹, Juan Li¹, Yao Chen¹, Yirou Wang¹, Xiaodong Huang¹, Yongnian Shen¹, Hao Zhang⁴, Jian Wang²*, Xiumin Wang¹*

¹ Department of Endocrinology and Metabolism, Shanghai Children's Medical Center, Shanghai JiaoTong University School of Medicine, Shanghai, China

² Department of Medical Genetics and Molecular Diagnostic Laboratory, Shanghai Children's Medical Center, Shanghai JiaoTong University School of Medicine, Shanghai, China

³ Department of Endocrinology and Genetic Metabolism disease, Children's Hospital of Chongqing Medical University, Chongqing, China

⁴ Department of Cardiothoracic Surgery, Heart Center, Shanghai Children's Medical Center, Shanghai JiaoTong University School of Medicine, Shanghai, China

# These authors contributed equally to the study.

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*Correspondence

Xiumin Wang
Department of Endocrinology and Metabolism, Shanghai Children's Medical Center, Shanghai Jiaotong University School of Medicine, Shanghai 200127, China. Email: wangxiumin@scmc.com.cn

Jian Wang
Department of Medical Genetics and Molecular Diagnostic Laboratory, Shanghai Children’s Medical Center, Shanghai Jiao Tong University School of Medicine, Shanghai, 200127 China. Email: labwangjian@shsmu.edu.cn

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Abstract

Context

Data of and studies based on exome sequencing for the genetic evaluation of short stature are limited, and more large-scale studies are warranted. Some factors increase the likelihood of a monogenic cause of short stature, including skeletal dysplasia, severe short stature, and small for gestational age (SGA) without catch-up growth. However, whether these factors can serve as predictors of molecular diagnosis remains unknown.

Objectives

We aimed to explore the diagnostic efficiency of the associated risk factors and their exome sequences for screening.

Design, Settings, and Patients

We defined and applied factors that increased the likelihood of monogenic causes of short stature in diagnostic genetic tests based on next-generation sequencing (NGS) in 814 patients with short stature and at least one other factor.
Results

Pathogenic/likely pathogenic (P/LP) variants in genes, copy number variations (CNVs), and chromosomal abnormalities were identified in 361 patients. We found P/LP variants among 111 genes, and RASopathies comprised the most important etiology. Short stature combined with other phenotypes significantly increased the likelihood of monogenic cause, including skeletal dysplasia, facial dysmorphism, and intellectual disability, compared with simple severe short stature (<–3 standard deviation scores). We report novel candidate pathogenic genes, KMT2C for unequivocal growth hormone insensitivity and GATA6 for SGA.

Conclusions

Our study identified the diagnostic characteristics of NGS in short stature with different risk factor. Our study provides novel insights into the current understanding of the etiology of short stature in patients with different phenotypes.

Keywords: short stature, whole exome sequencing, next generation sequencing
Introduction

Children who are over two standard deviations (>2 SD) below the population mean or the estimated familial target height are generally classified as having short stature and is a common reason for referrals to pediatric endocrinologists (1). Height in humans is influenced by hereditary, hormonal, nutritional, and environmental factors. Normal variations in adult height are largely attributed to the combined effects of various inherited genes. Thus, height is typically a polygenic trait (2–5). However, mutations in single genes can significantly affect height (6). Although several monogenic disorders can perturb growth, the role of genetic diagnostics in the evaluation of children with short stature has not reached a consensus.

With the use of next-generation sequencing (NGS) technology in clinical settings, genetic diagnostic strategies are playing increasingly important roles in determining the etiology and diagnosis of short stature. Genetic test algorithms might be useful for distinct diagnostic subgroups of patients with short stature (7). Exome sequencing has a high diagnostic yield for patients with short stature (8–9). However, data and studies based on exome sequencing for the genetic evaluation of short stature are limited, and more large-scale studies are warranted.
Factors such as severe familial forms of isolated growth hormone deficiency (IGHD) or specific syndromic forms of multiple pituitary hormone deficiencies (MPHD) increase the likelihood of a monogenic cause of short stature and severe short stature (<3 SD compared with the population mean or mid-parental target height), body disproportion and/or skeletal dysplasia, and small for gestational age (SGA) without adequate catch-up growth (6, 10). However, these factors have not been rigorously validated as predictors or indicators for genetic diagnoses.

We collected samples from 814 patients with suspected monogenic short stature and analyzed 330 of them by whole-exome sequencing (WES) and 484 using an inherited disease panel (ISD) (Figure 1). We defined factors that increased the likelihood of a monogenic cause of short stature and considered them as indications for genetic diagnosis. We conducted an in-depth analysis of NGS data of patients with short stature and different phenotypes. Our study provides insights into the current understanding of the etiologies of short stature.
Materials and Methods

Patient referral

We screened pathogenic variants in 814 children with short stature who were followed up between July 2015 and March 2020 in the Department of Endocrinology and Metabolism at Shanghai Children's Medical Center, Shanghai Jiaotong University School of Medicine and met the inclusion and exclusion criteria (Figure 1) (Supplement Methods in Reference 11).

The Ethics Committee of Shanghai Children's Medical Center approved the study. Written informed consent was obtained from the parents of all participants.

Health information and clinical history

The documented medical history included birth status, feeding habits, growth, development, and a history of illness of the children and their family members. Physical examinations included facial features, height, weight, head circumference, seated height, arm span, and signs of sex development.

Serum peak growth hormone (GH) level upon provocation (two independent provocation tests), and levels of insulin-like growth factor (IGF-1) (12,13), luteinizing hormone (LH), follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH), adrenocorticotropic hormone (ACTH), and cortisol
were determined using routine laboratory blood tests. Bone age was assessed by radiographic imaging and using the Greulich-Pyle Atlas method. Most patients were also assessed as needed by brain magnetic resonance imaging (MRI), echocardiography, gastrointestinal ultrasonography, and ultrasound of the urinary system.

**Molecular genetic analysis**

Peripheral blood samples were collected from the patients and their parents after obtaining written informed consent. Samples were analyzed by NGS and using the Agilent SureSelect capture technology (Agilent, Santa Clara, CA, USA), followed by either WES between 2018 and 2020, or an ISD (commercial version of Clearseq Inherited Disease panel from Agilent, part number: 5190-7519) comprising 2,742 genes between 2015 and 2017. The captured libraries were sequenced using the Illumina HiSeq 2500 system (Illumina, San Diego, CA, USA) and reads were aligned to the Human Reference Genome (NCBI build37, hg 19) using Burrows–Wheeler aligner-maximum exact matches (BWA-MEM) (14). Variants were called using the Genome Analysis Toolkit. All single nucleotide variants and indels were saved in variant call format (VCF) files and annotated using Ingenuity Variant Analysis (IVA) (Ingenuity Systems, Redwood City, CA, USA) and TGex (Translational Genomics Expert) platforms for variation filtering and interpretation (15). Briefly, all variants with a satisfactory sequencing depth and quality (average depth > 150, 20× coverage
> 98%) were filtered according to a minor allele frequency (MAF) of >0.01 in our in-house and genomAD exome (http://gnomad.broadinstitute.org/) databases (NGS sequencing data quality control metrics in Reference 11). The filtered variants were then sorted based on correlations between patient phenotypes and mutant genes using IVA and TGex. All suspected variants were confirmed by Sanger sequencing and validated using parental tests. Variants were manually classified according to the method recommended by the American College of Medical Genetics and Genomics (16).

Copy number variations were identified using open source CNVkit (17) software, which is a tool kit that can infer and visualize copy number from targeted DNA sequencing data. Previously aligned exome data (bam files) for sequencing variants screening were used again as input. Normal references for CNV identification were constructed based on sequencing data generated following the same protocol and experimental conditions from 10 normal males and 10 females who had no pathogenic CNVs, as validated by CMA. Individual CNVs were identified using default CNVkit settings. All CNVs identified using CNVkit were classified based on the CNV scoring metrics in ACMG/Clingen Technical Standards (18).
Statistical analysis

Fisher’s exact test was carried out for categorical variables between groups. Results with P < 0.05 were considered statistically significant. All analyses were performed using Statistical Package for the Social Sciences for Windows (version 23.0, SPSS, Inc., Chicago, IL, USA).

Results

Demographic data

The study involved 438 boys and 376 girls with a median age at diagnosis of 6.5 years (2 months to 17.68 years) and an average height SD of -3.043 (range: -2.01 to -8.53).

Among the 814 patients, samples of 330 and 484 with suspected monogenic short stature were respectively assessed using WES and the ISD. The P/LP variants in genes, CNVs, and chromosomal abnormalities, were identified in 361 patients (Figure 2). In addition, 279 patients harbored the P/LP variants distributed among 111 genes (Figure 3), 72 had P/LP CNVs, and 11 had P/LP chromosomal abnormalities (Figure 4).
Analysis of short stature with different phenotypes

Table 1 shows the diagnostic efficiency of NGS in patients with short stature and various phenotypes.

**IGHD, MPHD, and GHI**

Sixteen patients were diagnosed with severe IGHD based on clinical, laboratory, and imaging information, and a peak growth hormone (GH) level on provocation was <3 ng/mL. The P/LP variants were detected in 4 (25%) of 16 patients. Among 11 patients diagnosed with MPHD, 4 (36.4%) harbored the P/LP variants (Table 2). Unequivocal growth hormone insensitivity (GHI) was diagnosed in 39 patients with short stature based on peak GH ≥ 7 μg/L and IGF-1 SDS ≤ -2.0. Eight (20.5%) of the 39 patients had the P/LP variants (Table 3).

**SGA without catch-up growth**

Small for gestational age without catch-up growth at the age of 2 years was diagnosed in 87 patients with short stature, including 45 and 42 with and without syndromic causes. The P/LP variants were detected in 21 (24.1%) of these patients; the P/LP cases for short children with and without syndromic causes were 14 (31.1%) of the 45 causes and 7 (16.7%) of the 42 causes (Table 4).
Congenital anomalies (dysmorphic features), skeletal dysplasia, intellectual disability (developmental delay), and microcephaly

Among the 386 patients with short stature and congenital anomalies or dysmorphic features, the most prevalent were facial dysmorphism, disorders of sex development (DSD), and congenital heart disease (CHD) in 186 (48.2%), 96 (24.9%), and 93 (24.1%) of them, respectively. Figure 5a shows the intersection of pathogenic genes associated with these clinical features.

We identified the P/LP variants in 131 (70.4%) of 186 patients with facial dysmorphism (Supplementary Table 1 in Reference 11), in 16 (51.6%) of 31 with no other symptoms besides facial dysmorphism, and in 3 patients with these variants in the KMT2A gene. Among the 96 patients diagnosed with DSD, 70 and 26 were males and females, respectively, and the P/LP variants were detected in 51 (53.1% of them (Supplementary Table 2 in Reference 11). Thirty-nine male patients (46 XY) were diagnosed with cryptorchidism, and 26 (66.7%) of them harbored the P/LP variants (Supplementary Table 3 in Reference 11). Among 92 patients with CHD, 49 (53.3%) harbored P/LP variants (Supplementary Table 4 in Reference 11). Among 5 (20%) of 25 patients with short stature and CHD, P/LP variants were found in the NF1, PTPN11, and SHOC2 genes, and in two patients with 22q11.2 deletion syndrome (OMIM #611867).
Overall, 152 (64.7%) of 235 patients with skeletal dysplasia had P/LP variants. Pathogenic variants were identified in 59 genes and in 6 CNVs (Supplementary Table 5 in Reference 11). The P/LP variants detected in 98 (70.0%) of 140 patients with ID or DD were related to 34 genes in 50 (51.0%) of these patients. Seven patients were diagnosed with Cornelia de Lange syndrome (OMIM #122470) related to variants in four genes (NIPBL, HDAC8, SMC1A, and SMC3). Five patients harbored the most common pathogenic variant of KMT2A (Supplementary Table 6 in Reference 11). We identified CNVs in 48 (48.48%) of 98 patients (Supplementary Table 7 in Reference 11).

Figure 5b shows the intersections of pathogenic genes associated with congenital anomalies (dysmorphic features), skeletal dysplasia, and ID (DD). The P/LP variants were related to 6 genes and 4 CNVs in 9 (56.3%) of 16 patients with microcephaly (Supplementary Table 8 in Reference 11).

**Short stature and maternal history of recurrent miscarriages**

The mothers of three patients with short stature had experienced recurrent miscarriages. One of these patients had the P/LP variants comprising a 2q37.3 deletion and a 9q34.3 duplication, and one had a 22q11.21 deletion.
**Severe short stature (<-3 SD)**

We diagnosed 364 patients with severe short stature (<-3 SD compared with population mean or mid-parental target height) and 143 (39.3%) of them harbored P/LP variants. However, 143 of these patients had no other risk factors besides short stature (<-3 SD), whereas 16 (11.1%) of the 143 patients harbored the P/LP variants (Supplementary Table 9 in Reference 11).

**Unexpected findings with short stature cases**

We identified variants in genes (*GATA6*, *PLCB4*, and *RYR1*) that are not known to be related to short stature carried by patients 9990, 5260, and 9882 (Table 5). However, based on the type of variation, allele frequencies and other criteria, these variants could be classified into likely pathogenic groups. We assumed that these variants might contribute to our patients’ phenotypes, and the three genes could possibly be novel candidate genes responsible for short stature. However, due to the lack of evidence for certainty, we still regarded these situations as cases of uncertain diagnosis despite the pathogenicity classification.
Discussion

Growth is regulated by several genetic factors, but some individuals with significantly short stature harbor single-gene mutations that considerably affect height (19, 20). To accurately identify the etiology of short stature is challenging because extensive etiological heterogeneity and clinical complexity are involved. We identified factors that increased the likelihood of a monogenic cause of short stature and considered them as indications for genetic tests (Figure 1). We applied NGS to samples from 814 patients with suspected monogenic short stature and at least one of the factors listed in Figure 1. We identified 361 patients with P/LP variants by NGS in our study, and the P/LP variants were distributed among 111 genes; RASopathies caused by mutations in genes of the Ras-MAPK pathway comprised the most important etiology of short stature in our cohort (Figure 3). The CNVs diagnosed using NGS mostly caused 22q11.2 and 7q11.23 deletion syndromes. Our patients were of short stature with a risk factor, and the diagnosis yield for monogenic diseases was higher than that in the general group of children with short stature.

Genetic defects of the GH–IGF-1 axis have been associated with severe IGHD and MPH (21). Our findings showed that variants in GH1 constitute a major cause of severe IGHD. Variants in GLI2 were detected in 3 of 11 patients with MPH. Serum peak GH level on provocation in positive IGHD and MPH patients was <1 ng/mL.
Classical GHI originally described by Laron et al. in 1966 (22, 23) and called Laron-type dwarfism or Laron syndrome (OMIM #262500) is caused by a defect in the GH receptor (GHR) gene, resulting in extreme GH resistance and an associated IGF-1 deficiency (24). This rare and extreme phenotype became synonymous with a diagnosis of GHI. During the past 20 years, the GHI categories have been expanded to include mild or moderate GHI and several other congenital and acquired conditions associated with it (25). Among our patients with GHI, 20.51% harbored pathogenic variants, of which PTPN11 was the most common. Studies have suggested that the constitutively activated RAS-MAPK pathway in Noonan syndrome (OMIM #163590) and other RASopathies can lead to inhibition of the JAK/STAT pathway, relatively low levels of IGF-I, and subsequently short stature (26). The most common mutation affects PTPN11, which encodes the cytoplasmic SH2 domain-containing protein tyrosine phosphatase 2 (SHP-2). This enzyme dephosphorylates STAT5b, consequently activating mutations of PTPN11 and downregulating STAT5b activity, while activating the MAPK pathway. The growth response to GH is lower in individuals who are PTPN11 variant-positive than those who are negative (27). Our findings suggested that GHI is most likely caused by variants in PTPN11. We identified a patient with GHI pathogenic variants of KMT2C. KMT2C encodes a histone methyltransferase that regulates gene transcription by modifying chromatin structure. A heterozygous mutation in KMT2C is associated with Kleefstra syndrome-2,
(OMIM #617768), which is a rare genetic syndrome with delayed psychomotor development, variable intellectual disability, and mild dysmorphic features. Some patients have short stature, but the involvement of the GH-IGF-1 axis is unknown (28, 29, 30). Our findings suggested that the limited growth of patients with a heterozygous mutation in KMT2C can be attributed to an IGF-1 deficiency.

The process of human fetal growth is regulated by fetal and maternal genetic factors that affect the intrauterine environment to ensure effective nutrient exchange between the mother and fetus via the placenta. Small for gestational age has been defined either as being below the 10th percentile for weight at a given gestational age or as having a birth length or weight SD < 2.0 (below the 2.3 percentile) (31). Among the causes of SGA are maternal health and obstetric factors, placental insufficiency, and fetal genetic factors. Among children with idiopathic SGA, ~85% catch up to the 3rd percentile of length by the age of 2 years (32, 33). Children without catch-up growth require further evaluation, especially a subset with progressive postnatal growth failure. The diagnostic yield of NGS in SGA in the present study was 21 (24.1%) of 87, among whom 13 (14.9%) and 8 (9.2%) had P/LP variants in genes and CNVs, which was below that of the total cohort (361 [44.3%] of 814) (P <0.05). Imprinted genes in the placenta are important for the control of fetal growth (34-35). A recent study of 269 patients with SGA with short stature reported a diagnostic yield of
107 (39.78%) of the 269 patients by comparative genomic hybridization combined with methylation analysis, and 32.34% (87/269) patients were diagnosed with imprinting disorders and 7.44% (20/269) were CNVs (35). The diagnostic power of exome sequencing in SGA is limited, further methylation analysis can be an effective approach to diagnose SGA, and environmental causes for SGA should be considered.

One patient with SGA, CHD, and diabetes harbored pathogenic variants in GATA6, which encodes GATA-binding protein 6 and has not yet been associated with short stature. GATA6 belongs to a small family of zinc finger transcription factors that play important roles in the regulation of cellular differentiation and organogenesis during development in vertebrate. The GATA6 phenotypic spectrum includes neonatal-, childhood-, and adult-onset diabetes; exocrine pancreatic insufficiency; pancreatic agenesis or hypoplasia; various cardiac malformations, hypothyroidism, hypopituitarism and pituitary agenesis; intestinal malrotation; hernias; colonic perforation; structural kidney abnormalities; neurocognitive deficits; and seizures (36, 37, 38). Two patients with pathogenic variants in GATA6 had intrauterine growth restriction (IUGR) (39, 40). Thus, GATA6 may be a candidate pathogenic gene for SGA without catch-up growth.

RASopathies were the most important etiology of short stature in patients with CHD (Supplementary Table 4 in Reference 11). The P/LP variants were
detected in 20% of the short stature patients who presented with no other symptoms except CHD, and 22q11.2 deletion syndrome was the most common pathogenic variant. The clinical presentation of 22q11.2 deletion syndrome varies by age, and clinical complexity might pose challenges in accurate diagnoses (41). Next-generation sequencing should facilitate the earlier detection and increased recognition of 22q11.2 deletion syndrome.

We detected P/LP variants in 51 (53.1%) of the 96 patients with short stature and DSD. Thirty-nine males (46 XY) had cryptorchidism and 26 (66.7%) of the 39 patients harbored the P/LP variants. Cryptorchidism (OMIM #219250) is one of the most frequent congenital birth defects in boys and appears in 2%–4% of full-term male births (42). Maldescent testicles can be an isolated event or result from a variety of syndromes (syndromic cryptorchidism) and other non-syndromic diseases (non-syndromic cryptorchidism) (43, 44, 45). Data from 50 studies have associated cryptorchidism with 44 syndromes, as well as genomic loci include 38 protein-coding genes and 22 structural variations containing microdeletions and microduplications (46). Our findings suggest that short stature combined with cryptorchidism considerably increases the likelihood of a monogenic cause of short stature.

Geneticists identified facial dysmorphism in 186 patients in our cohort, and we detected P/LP variants related to 52 genes in 131 (70.4%) of the patients. Many syndromes have recognizable facial features, and Face2gene has
achieved a high diagnostic rate in genetic diseases based on facial images (47). Our findings suggested that short stature combined with facial dysmorphism indicates a need for genetic testing. The P/LP variants were detected in 16 (51.6%) of the 31 patients who presented with no other symptoms except facial dysmorphism. Three patients harbored the P/LP variants in KMT2A.

Wiedemann–Steiner syndrome (OMIM #605130) is a rare genetic disorder characterized by facial gestalt, neurodevelopmental delay, skeletal anomalies, and growth retardation, which is caused by variations in KMT2A (48). Most patients exhibited suggestive features, but characteristics were less obvious in others (49). Wiedemann–Steiner syndrome is an important consideration for short stature alone with facial dysmorphism.

In our study, 152 (64.7%) of the 235 patients with skeletal dysplasia harbored the P/LP variants related to 59 genes and 6 CNVs (Supplementary Table 5 in Reference 11). Skeletal dysplasia features, mainly attributable to variants in protein-coding genes, rarely involve structural variations. MFN2, RYR1, and PLCB4 have not been associated with short stature in previous reports; patient phenotypes, types of variations, allele frequencies, and other criteria could classify variants into P/LP groups. Variants in MFN2 or RYR1 lead to a slow, progressive development of neuromuscular disorders, and clinical manifestations include skeletal deformities (50, 51). Pathogenic variants in PLCB4 are associated with auriculocondylar syndrome (OMIM #602483),
which is mainly characterized by micrognathia, a small mandibular condyle, facial asymmetry, and question mark-shaped ears. It is a rare disease that segregates in an autosomal dominant pattern in most of the families described in the literature with evident intrafamilial variability (52, 53).

Both DD and ID affect 1%–3% of children and a genetic etiology is involved in approximately 50% of those affected (54). Our findings suggested that DD and ID combined with short stature increased the likelihood of a monogenic cause, and structural variations containing microdeletions and microduplications were major causes of these conditions. Cornelia de Lange, Wiedemann-Steiner, and Williams-Beuren (OMIM #194050) syndromes are common pathologies (Supplementary Table 6, Supplementary Table 7 in Reference 11).

Microcephaly is defined as a head circumference of > 2 SD below the mean for gender and age. Growth retardation accompanied by microcephaly is mainly associated with microcephalic primordial dwarfism such as Cornelia de Lange, MOPD I (OMIM #210710), MOPD II (OMIM #210720), Seckel (OMIM #210600), and Meier-Gorlin (OMIM #224690) syndromes (20). Our findings showed an extremely high positive diagnostic yield for microcephaly with mental retardation, and syndromes associated with abnormal DNA repair, such as Bloom (OMIM #210900) and Cockayne (OMIM #216400, #133540) syndromes, should be recognized (Supplementary Table 8 in Reference 11).
A recent study diagnosed a pathological cause of severe short stature (< -3 SD compared with the population mean) in 76% and 71% of girls and boys investigated, but a genetic cause of severe short stature was not determined (55). For severe short stature without other symptoms, genetic defects affecting paracrine factors in the growth plate (FGFR3, GNAS, and IHH), genetic defects affecting the cartilage extracellular matrix (ACAN), genetic defects affecting the GH-IGF1-IGF1R axis (GHRHR, GHSR, and IGF1R), and Wiedemann–Steiner syndrome (KMT2A) with fewer characteristics should be carefully analyzed.

In conclusion, NGS combined with risk factor screening significantly increased the diagnostic yield of patients with short stature. The diagnostic power of exome sequencing in children with SGA is limited, and adding methylation studies can be an effective approach to diagnose children with SGA. Variants in PTPN11 might comprise the main etiology of mild GHI, and further investigation should target the effectiveness of recombinant human growth hormone (rhGH) therapy for patients with Noonan syndrome and IGF-1 therapy may be an appropriate therapy for these patients. Short stature with facial features indicates the possibility of a genetic etiology, even if accompanied by a single symptom. Some of the patients in this study harbored the P/LP variants in GATA6, RYR1, and PLCB4 that have not yet been associated with short stature. Based on phenotypes, types of variations, allele
frequencies, and other criteria, gene variants can be classified into P/LP groups. Short stature might also be a non-primary component of a few syndromic disorders, and WES presents a higher diagnostic yield than short stature panels for these conditions.

**Limitations**

Our study had some limitations. This study was performed in one institute with a large referral population, which could have created a selection bias that likely increased the diagnostic yield of WES in this study. Some children with short stature may have been already diagnosed either clinically or genetically and hence were ineligible for the study, such as those with achondroplasia (OMIM #100800). Some patients were not assessed using WES and rare CNVs are difficult to diagnose using NGS. Although CNV detection based on read-depth information from WES data has been widely adopted in clinical practical, the discovery rate of rare and non-recurrent CNVs still largely depends on principle of the algorithm, quality of the raw sequencing data, and number of samples in the same batch (56). Future research should further expand the survey sample and improve testing methods.
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Data availability

Data are available from the corresponding author on reasonable request.

Ethics Declaration

The Ethics Committee at Shanghai Children's Medical Center approved the study. Written informed consent was obtained from the parents of all participants.
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Legends for tables and figures

Table 1. The diagnostic efficiency of NGS in short stature patients with different phenotypes.

IGHD: isolated growth hormone deficiency; MPHD: multiple pituitary hormone deficiencies; GHI: unequivocal growth hormone insensitivity; SGA: small for gestational age; SDS: standard deviation scores; CNV: Copy number variation; P: The Fisher’s exact test was carried out for categorical variables between different phenotypes and height below -3SD (None of additional phenotypes).

Table 2. The phenotype and genotype analysis of patients with IGHD and MPHD. 25% (4/16) patients with severe IGHD were identified with pathogenic/likely pathogenic variants in two genes (GH1, SOX3). 36.36% (4/11) patients with MPHD were identified with pathogenic/likely pathogenic variants in two genes (GLI2, NPHP4).

IGHD: isolated growth hormone deficiency; MPHD: multiple pituitary hormone deficiencies; SDS: standard deviation scores; F: paternal inheritance; M: maternal inheritance; F/M: inherited respectively from parents; LH: Luteinizing hormone; FSH: Follicle stimulating hormone; TSH: Thyroid stimulating hormone; ACTH: Adrenocorticotropic hormone
Table 3. 20.51% (8/39) patients with unequivocal GHI were identified with pathogenic/likely pathogenic variants.

GHI: growth hormone insensitivity; CHD: congenital heart disease; F: paternal inheritance; M: maternal inheritance; F/M: inherited respectively from parents; NA: Not available

Table 4. 24.1% (21/87) SGA without catch-up growth after 2 years of birth were identified with pathogenic/likely pathogenic variants.

SGA: small for gestational age; CHD: congenital heart disease; F: paternal inheritance; M: maternal inheritance; F/M: inherited respectively from parents; NA: Not available; het: heterozygote; hom: homozygote

Table 5. Unexpected findings with short stature cases and novel candidate genes. Pathogenic variants in genes that are not known to be related to short stature (GATA6, PLCB4, RYR1) were identified in patients 9990, 5260 and 9882.

SGA: small for gestational age; CHD: congenital heart disease; F: paternal inheritance; het: heterozygote; LP: likely pathogenic

Figure 1. Flowchart of patients recruitment and variants discovery approach.

SDS: standard deviation scores; WES: whole exome sequence
Figure 2. a. 44.3% (361/814) patients were identified with pathogenic/likely pathogenic variants, WES was 46.4% and that of panel was 43.0%. b. 361 patients harbored P/LP variants, including 77.0% patients harbored with variants in genes, 19.7% harbored with copy number variations, 3.0% harbored with chromosomal abnormalities and 0.3% harbored copy number variations combined variants in genes.

WES: whole exome sequence; Panel: inherited disease panel

Figure 3. A total of 279 patients were identified with pathogenic/likely pathogenic variants distributed among 111 genes, these genes were classified centred on the epiphyseal growth plate.

Figure 4. a. 72 patients had identified with pathogenic/likely pathogenic copy number variations, 22q11.2 deletion syndrome was most common copy number variations. b. 11 patients had pathogenic/likely pathogenic chromosomal abnormalities.

Figure 5. The intersection of pathogenic genes associated with different clinical features. a. 70.4% (131/186) of the patients with facial dysmorphism were identified with P/LP variants, related to 52 genes. 53.1% (51/96) of the patients with disorders of sex development were identified with P/LP variants, related to 25 genes. 53.3% (49/92) of the patients with congenital heart disease,
related to 14 genes. The intersection of pathogenic genes of these clinical features related to 5 genes, including PTPN11, RAF1, SOS1, NIPBL and KMT2A. b. 56.2% (217/386) patients with congenital anomalies or dysmorphic features were identified with pathogenic/likely pathogenic variants, related to 76 genes. 64.7% (152/235) of the patients with skeletal dysplasia had pathogenic/likely pathogenic variants, related to 60 genes. 70.0% (98/140) of the patients with intellectual disability or developmental delay were identified with pathogenic/likely pathogenic variants, related to 34 genes. The intersection of pathogenic genes of these clinical features related to 12 genes, including PTPN11, RAF1, HRAS, CLCN7, TWIST1, HDAC8, ANKRD11, OFD1, IDS, ERCC6, FAM111A and FGFR3.
Table 1. The diagnostic efficiency of NGS in short stature patients with different phenotypes.

| Phenotype                                                                 | No. of patients | P/LP cases (%) | Variants in genes | CNVs | Chromosomal abnormalities | CNVs combined variants in genes | P     |
|---------------------------------------------------------------------------|-----------------|----------------|-------------------|------|--------------------------|---------------------------------|-------|
| Severe IGHD                                                               | 16              | 4(25%)         | 4                 | /    | /                        | /                               | 0.121 |
| MPHD                                                                      | 11              | 4(36.4%)       | 4                 | /    | /                        | /                               | < 0.001 |
| GHI                                                                       | 39              | 8(20.5%)       | 6                 | 2    |                          | /                               | < 0.001 |
| SGA without catch-up growth congenital anomalies or dysmorphic features  | 87              | 21(24.1%)      | 11                | 9    |                          | 1                               | < 0.001 |
| SGA without catch-up growth congenital anomalies or dysmorphic features  | 387             | 217(56.2%)     | 162               | 45   | 10                       | /                               | < 0.001 |
| Skeletal dysplasia                                                        | 235             | 152(64.7%)     | 146               | 6    |                          | /                               | < 0.001 |
| Intellectual disability or developmental delay                           | 140             | 98(70%)        | 50                | 48   |                          | /                               | < 0.001 |
| Microcephaly                                                              | 16              | 9(56.3%)       | 6                 | 3    |                          | /                               | 0.003 |
| Mother with recurrent miscarriage height below -3SD (None of additional phenotypes) | 3               | 2(66.7%)       | /                 | 2    |                          | /                               | 0.312 |
| microcephaly                                                              | 143             | 16(11.2%)      | 12                | 3    | 1                        | /                               | (Ref.) |

IGHD: isolated growth hormone deficiency; MPHD: multiple pituitary hormone deficiencies; GHI: unequivocal growth hormone insensitivity; SGA: small for gestational age; SDS: standard deviation scores; CNV: Copy number variation;
P: The Fisher’s exact test was carried out for categorical variables between different phenotypes and height below -3SD (None of additional phenotypes).
Table 2. The phenotype and genotype analysis of patients with IGHD and MPHD. 25% (4/16) patients with severe IGHD were identified with pathogenic/likely pathogenic variants in two genes (GH1, SOX3). 36.36% (4/11) patients with MPHD were identified with pathogenic/likely pathogenic variants in two genes (GLI2, NPHP4).

IGHD: isolated growth hormone deficiency; MPHD: multiple pituitary hormone deficiencies; SDS: standard deviation scores; F: paternal inheritance; M: maternal inheritance; F/M: inherited respectively from parents; LH: Luteinizing hormone; FSH: Follicle stimulating hormone; TSH: Thyroid stimulating hormone; ACTH: Adrenocorticotropic hormone

| Patient | Age (year) | Height (SDS) | GH Peak (ng/ml) | Other pituitary Hormone | Other Phenotypes | MRI | Gene | Variation | Parental validation |
|---------|------------|--------------|----------------|--------------------------|-----------------|-----|------|-----------|---------------------|
| 61 Mal | 15.50      | -5.64        | 0.56           | Normal                   |                 |     | Normal | NM_0005   | F/M c.242_243 del p.(Ser81*) |
| 65 Mal | 3.92       | -3.17        | 0.01           | Normal                   | Cryptorchidism  |     | Small pituitary size | NM_0005   | F/M c.291+1G>A p.? |
| 10 Mal | 2.83       | -8.54        | 0.06           | Normal                   | Big and protruding foreheads |     | Small pituitary size | NM_0005   | F/M [c.240del]/[Exon1-5 del] p.(Ser81G |
| 39  | Male | 11.18 | -0.94 | 0.11 | Normal | Small penis, Mild learning difficulties | Anterior pituitary hypoplasia | SO X3 | NM_0056 | M | c.424C>A p.(Pro142 Thr) |
|------|------|-------|-------|-------|--------|------------------------------------|-------------------------------|-------|----------|---|--------------------------|
| 51   | Male | 2.56  | -5.3  | 0.45  | LH↓, FSH↓, TSH↓ | Micropenis, small testes | Anterior pituitary hypoplasia | GL I2 | De novo | NM_0052 | 70.4: | c.3463_3464del p.(Asp1155Argfs*39) |
| 55   | Male | 2.25  | -5.75 | 0.04  | LH↓, FSH↓, TSH↓, ACTH↓ | Micropenis, small testes, polydactyly | Anterior pituitary hypoplasia | GL I2 | | NM_0052 | 70.4: | c.3137del p.(Gly1046Alafs*84) |
| 66   | Male | 5.90  | -4    | 0.52  | LH↓, FSH↓, TSH↓ | Micropenis, small testes, deafness, intellectual disability | Anterior pituitary hypoplasia | GL I2 | | NM_0052 | 70.4: | c.3640C>T p.(Gln1214*) |
| 39   | Male | 12.72 | -4.66 | 0.08  | TSH↓, ACTH↓ | Hematuria, normal renal function | Anterior pituitary hypoplasia | NP H P4 | | NM_0151 | 02.4: | c.3196C>T p.(Gln1066*) |
Table 3. 20.51% (8/39) patients with unequivocal GHI were identified with pathogenic/likely pathogenic variants.

GHI: growth hormone insensitivity; CHD: congenital heart disease; F: paternal inheritance; M: maternal inheritance; F/M: inherited respectively from parents; NA: Not available

| Patient | Sex | Age (year) | Height (SDS) | GH peak (ng/ml) | IGF-1 (SDS) | Other phenotypes | Variation | Parental validation |
|---------|-----|------------|--------------|----------------|-------------|------------------|-----------|---------------------|
| 435     | Fem | 10.08      | 3.37         | 19.71          | < -2SD S    | CHD, Facial dysmorphisms, pectus excavatum | PTPN11 NM_002834.3: c.1510A>G p.(Met504Val) | NA |
| 839     | Fem | 8.5        | 4.35         | 13.06          | < -2SD S    | CHD, Facial dysmorphisms, pectus excavatum, amblyopia, deafness | PTPN11 NM_002834.3: c.218C>T p.(Thr73Ile) | De novo |
| 895     | Mal | 11.67      | 4.48         | 8.87           | < -2SD S    | CHD, Facial dysmorphisms, pectus excavatum, cryptorchidism | PTPN11 NM_002834.3: c.923A > G p.(Asn308Ser) | M |
| 859     | Fem | 12.33      | 3.54         | 10             | < -2SD S    | CHD, Webbed neck HP:0000465 | PTPN11 NM_002834.3: c.188A>G | De novo |

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| Patient | Gender | Age | Height | Z-Score | Condition | Gene | Mutation | sex | 2SDS | Remarks |
|---------|--------|-----|--------|---------|-----------|-------|----------|------|-------|----------|
| 222     | Male   | 12.09 | 2.51   | 9.13    | Subclinical hypothyroidism | DUOX2 | NM_014080.4: [c.3329G>A]/[c.1310 G>C] [p.(Arg1110Gln)]/[p.(Gly437Ala)] | F/M |     |          |
| 131     | Female | 11.14 | 2.05   | 10.2    | Primordial uterus, congenital spina bifida | KMT2C | NM_170606.3: De novo | c.3841+1G>A | p.? |          |
| 576     | Female | 11.25 | 3.52   | 9.84    |                        |        | dup(16)(q11.2)(over 300kb) | NA |     |          |
| 761     | Male   | 8.3   | 3.09   | 10.73   | CHD                   | CHD   | del(22)(q11.21) [hg19(chr22:18,900,287 -21,245,501)] (over 2300kb) | NA |     |          |
Table 4. 24.1% (21/87) SGA without catch-up growth after 2 years of birth were identified with pathogenic/likely pathogenic variants.

SGA: small for gestational age; CHD: congenital heart disease; F: paternal inheritance; M: maternal inheritance; F/M: inherited respectively from parents; NA: Not available; het: heterozygote; hom: homozygote

| Patient | Sex | Age (year) | Height (SDS) | Phenotypes | Variation |
|---------|-----|------------|--------------|------------|-----------|
| 5341    | Female | 5.00       | 3.33         | SGA, CHD, facial dysmorphicis, development delay | KMT2A NM_001197104.1: c.11716C>T p.(Arg3906Cys) (het) (De novo) |
| 6533    | Female | 6.50       | -2           | SGA        | COL1A1 NM_000088.3: c.1171G>A p.(Asp391Asn) (het) (De novo) |
| 4042    | Male   | 4.43       | -4.02        | SGA        | COL2A1 NM_001844.4: c.1016G>A p.(Gly339Asp) (het) (De novo) |
| 5621    | Female | 16.3       | 1.31         | SGA, Cleft lip and palate, DSD, no olfactory bulb | FGFR1 NM_023110.2: c.760C>T p.(Arg254Trp) (het) (De novo) |
| WJ-584  | Male   | 11.0       | 2.64         | SGA, facial dysmorhisms, microtia, absence of patella | ORC6 NM_014321.3: c.67A>G p.(Lys23Glu) (hom)(F/M) |
| WJ-656  | Male   | 13.3       | 5.09         | SGA, facial dysmorphicis, microcephaly, development delay, acanthosis nigricans type 2 diabetes | PCNT NM_006031.5: [c.3103C>T][c.502C>T][p.(Arg1035*)][p.(Gln168*)] (compound heterozygote) (F/M) |
| 8816    | Male   | 4.50       | 2.38         | SGA, CHD, | ANKRD11 NM_013275.5:c.3140_3143del p.(Gln1047Argfs*270) (het)(M) |
| 7290    | Male   | 4.83       | 3.83         | SGA        | RPS7 NM_021140.3:c.75+2T>C p.? (het) (NA) |
| 9021    | Female | 7          | -2.4         | SGA        | POC1A NM_015426.4: c.981+1G>A p.? (hom)(F/M) |
| 9153    | Female | 3.92       | -2.3         | SGA        | CASR NM_000388.3: c.3082C>T p.(Gln1028*) (het)(M) |
| 6500    | Female | 5.00       | 3.25         | SGA, DSD  | GHR NM_000163.4: c.136+1G>A p.? (hom)(F/M) |
| Sample ID | Gender | Age | Z Score | Growth Status | SOX11 Mutations | Other Chromosomal Abnormalities |
|-----------|--------|-----|---------|---------------|-----------------|--------------------------------|
| 7500      | Male   | 3.00| -4.78   | SGA           |                 |                                |
| 1392      | Female | 5.83| -3.98   | SGA, IGF-1>2SD|                 |                                |
| 1369      | Male   | 10.0| -1.9    | SGA, intellectual disability | del(7)(q11.23)[hg19,(chr7:73,442,119-74,175,022)] (over 700kb) |                                |
| 1085      | Female | 7.67| -5.8    | SGA, CHD, facial dysmorphisms, intellectual disability | del(18)(p11.31-p11.21)[hg19,(chr18:2,916,992-12,884,236)] (over 9900kb) |                                |
| 1272      | Female | 1.50| -4.1    | SGA, Facial dysmorphisms, development delay | del(7)(q36.1-q36.3)[hg19,(chr7:150,642,044-157,210,133)] (over6500kb) | dup(18)(q23)[hg19,(chr18:77,439,801-77,514,510)] (over 200kb) |
| 2882      | Female | 6.08| -3.35   | SGA, CHD, facial dysmorphisms, intellectual disability, auricle deformity | del(9)(q21.11-q21.31)[hg19,(chr9:71000154-83236029)] (12236kb) |                                |
| 7767      | Female | 6.58| -4.93   | SGA, CHD, intellectual disability | del(13)(q31.1-q32.1)[hg19,(chr13:69,374,377-101,164,769)] (over 3200kb) |                                |
| 7177      | Male   | 7.00| -1.9    | SGA | del(15)(q26.3)[hg19,(chr15:65,413,208-69,731,844)] (over 2600kb) |                                |
| 9951      | Female | 1.50| -2.5    | SGA, facial dysmorphisms, development delay | del(16)(p13.11)[hg19,(chr16:15,737,124-16,317,328)] (over 500kb) |                                |
| 1372      | Female | 7.00| -2.9    | SGA, development delay | dup(19)(p13.3)[hg19,(chr19:852,303-6,720,661)] (over 5800kb) |                                |
Table 5. Unexpected findings with short stature cases and novel candidate genes. Pathogenic variants in genes that are not known to be related to short stature (*GATA6*, *PLCB4*, *RYR1*) were identified in patients 9990, 5260 and 9882.

SGA: small for gestational age; CHD: congenital heart disease; F: paternal inheritance; het: heterozygote; LP: likely pathogenic

| Patient | Sex | Age (year) | Height (SDS) | Phenotypes | Variation | ACMG category |
|---------|-----|------------|--------------|------------|-----------|---------------|
| 9990    | Male | 2          | -2.2         | SGA, CHD, type 1 Diabetes | *GATA6* NM_005257.5: c.1366C>T p.(Arg456Cys) (het) (De novo) | LP |
| 5260    | Male | 8          | -3.01        | facial asymmetry, development delay | *PLCB4* NM_000933.3: c.2980delA p.(Met994*) (het)(F) | LP |
| 9882    | Male | 3.4        | -3.41        | pectus excavatum, scoliosis, cryptorchidism | *RYR1* NM_000540.2: c.7523G>A p.(Arg2508His) (het) (De novo) | LP |
Figure 1

Short stature patients recruitment
Height SDS < -2

Inclusion criteria:
- presence of one or more of the additional factors: severe isolated growth hormone deficiency (IGHD), multiple pituitary hormone deficiency (MPHPD), unequivocal growth hormone insensitivity (GHI), small for gestational age (SGA) without catch-up growth, additional congenital anomalies or dysmorphic features, evidence of a skeletal dysplasia, associated intellectual disability (ID) or developmental delay (DD), microcephaly, mother with recurrent miscarriage, height below -3SDS

Exclusion criteria:
- patients with clinically diagnosable conditions: such as Down’s syndrome, Turner syndrome with typical phenotypes (confirmed by karyotyping), pituitary tumor, short stature secondary to chronic illness, with definitive genetic diagnosis

814 patients enrolled in the study

Molecular genetic analysis
- 330 by WES
- 484 by inherited disease panel

Analysis of exome sequencing in short stature with different phenotypes
Figure 4

a) 22q11.2 deletion syndrome (1983.3)
   T6q11.23 deletion syndrome (140560)
   2q14.2q26 microdeletion syndrome
   Chromosome 18p deletion syndrome (140390)
   Miller–Ozgur/lliasencephaly syndrome (247200)
   Other
   69.73%

b) 45X
   45X/46XY mosaicism
   Large deletion of Xp and large duplication of Xq
   47,XXY
   54.55%

