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Molecular diagnosis is an important indicator for response to growth hormone therapy in children with short stature

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ABSTRACT

Background: Significant differences have been observed in the efficacy of recombinant human growth hormone (rhGH) treatment for short children. The present study aimed to identify the genetic etiology of short stature and to assess the role of molecular diagnosis in predicting responses to rhGH treatment.
Methods: A total of 407 short children were included in the present study, 226 of whom received rhGH treatment. Whole-exome sequencing (WES) was conducted on short children to identify the underlying genetic etiology. Correlations between molecular diagnosis and the efficacy of rhGH treatment were examined.
Results: Pathogenic or likely pathogenic mutations were identified in 86 of the 407 patients (21.1%), including 36 (41.9%) novel variants. Among the multiple pathways affecting short stature, genes involved in fundamental cellular processes (38.7%) play a larger role, especially the RAS-MAPK pathway. In general, patients without pathogenic mutations responded better to rhGH than those with mutations. Furthermore, patients with hormone signaling pathway mutations had a better response to rhGH, while those with paracrine factor mutations had a worse response to rhGH.
Conclusions: This study highlights the utility of WES in identifying genetic etiology in children with short stature. Identifying likely causal mutations is an important factor in predicting rhGH response.

1. Introduction

Pediatric endocrinologists often encounter cases of short stature as one of the most prevalent symptoms. The evaluation of short stature patients involves comprehensive assessments encompassing clinical, phenotypic, physiological, and biochemical evaluations, along with genetic analysis targeted toward patients suspected of having specific syndromes [1,2]. It is believed that genetic factors contribute to 80% of the variability in human height and are considered the primary reason for differences in individual growth [3]. Short stature can be attributed to a singular dominant genetic factor in certain cases. Notable examples include Noonan syndrome (NS), Turner syndrome (TS), and neurofibromatosis type 1 (NF-1) [4,5]. However, in approximately 60–80% of patients, the etiology remains unclear, preventing early and precise treatment of growth retardation and coexisting disorders, as well as adequate genetic counseling [6,7]. Advances in molecular technology and bioinformatics have made genetic research available to clinicians and revealed many genetic causes of growth failure. The successful application of whole-exome sequencing (WES) has led to the identification of coding mutations responsible for monogenic growth disorders. Using appropriate genetic testing methods, approximately 25–40% of children with unexplained short stature can receive a molecular diagnosis, shedding light on the underlying cause of their condition [8].

Recombinant human growth hormone (rhGH) treatment has been proven to effectively improve stature in individuals with growth disorders; however, there is significant variability in the response to rhGH, both between different types of growth disorders and between individuals with the same etiology of short stature [9,10]. This suggests

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that factors that differ among patients, including genetic mutations, may contribute to the rhGH response. Specific models including multiple clinical variables to predict patient response to rhGH have been developed. However, these clinical parameters only partially predict rhGH treatment responses [11]. Individual genetic variations also play a crucial role in the response to rhGH treatment, as different individuals have varied genotypes that can affect their responsiveness to rhGH. Furthermore, identifying genetic variants that affect this response may reveal the biological mechanisms underlying the differences in rhGH treatment response. In addition, a previous study revealed that approximately 40% of the variations in height during normal growth can be attributed to the correlation between mid-parental height (which serves as a proxy for genetic factors) and the final height of offspring after accounting for sex and age differences [12]. Hence, the objective of this study was to evaluate the genetic basis underlying growth disorders in children with short stature while also elucidating their clinical features and the efficacy of rhGH therapy in improving their condition.

2. Methods

2.1. Patients

The study group was composed of 407 unrelated short children admitted to the Department of Endocrinology at the Affiliated Hospital of Jining Medical University between March 2013 and May 2020. All subjects underwent clinical evaluation, laboratory examination and imaging assessment. Among them, 226 subjects received rhGH treatment and were followed up. The study enrolled participants who met specific inclusion and exclusion criteria. Eligible participants were children or adolescents between the ages of 3 and 18 years who had a height more than two standard deviations (SDs) below the average for individuals of the same race, age, and sex. However, individuals who had perfect genetic testing to identify genetic etiologies, such as short stature due to NS, TS, or Prader-Willi syndrome, were excluded from the study.

The study received ethical approval from the Human Ethics Committee of the Affiliated Hospital of Jining Medical University. All participants or their parents provided written informed consent.

2.2. Clinical indicator assessment

Birth indicators, including birth length and birth weight, were obtained from the patient's parents and recorded. The height and weight of the patients and their parents were measured, and the stage of puberty was assessed according to the patients' presentation of secondary sexual characteristics. The height SDS was determined by comparing it to the normal range for Chinese children [13]. The height of the fathers and mothers of all patients was also measured, and mid-parental height (MPH) was calculated [14]. The laboratory examination included the assessment of GH peaks through two GH stimulation tests, as well as the detection of insulin-like growth factor 1 (IGF-1). The specific measurement and detection methods have been described in previous reports [15]. IGF-1 SDS was calculated by comparing the IGF-1 levels of the participants to those of healthy children and adolescents [16]. The assessment of left hand and wrist bone age (BA) using the Greulich and Pyle method was performed using the Ysio SIEMENS X-ray machine from Germany [17]. All of the patients underwent brain magnetic resonance imaging.

2.3. Genetic analysis

DNA samples from all patients were used to construct libraries using the Agilent liquid capture system, and whole exome sequencing was performed on the BGISEQ-500 platform, with an average sequencing depth of 100× and 98% coverage above 20-fold, with paired-end reads of 150 bp. Reads were aligned to the human reference genome GRCh37/hg19. Data and bioinformatics analyses were performed according to methods described in previous studies [15]. The validation of discovered candidate variations was carried out utilizing Sanger sequencing. In compliance with the guidelines established by the American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP), the degree of pathogenicity for the genetic alterations identified was assessed [4].

The identification of copy number variations (CNVs) was accomplished using CNVkit software, an open-source tool [18]. To confirm the presence of the identified candidate variants, CNV-seq analysis was performed. The default settings of the CNVkit software were employed to identify individual copy number variations (CNVs). Subsequently, all CNVs identified utilizing CNVkit were categorized based on the CNV scoring metrics outlined in the ACMG/Clingen Technical Standards [19].

2.4. Statistical analysis

The presentation of continuous variables was achieved via the mean ± standard deviation, whereas categorical variables were expressed using numerical counts and percentages. Student's *t* test was employed to detect dissimilarities between two groups for variables that followed a normal distribution, whereas for variables that did not meet the condition of normal distribution, the Kruskal–Wallis test was used. Furthermore, the chi-square test and Fisher's exact test were conducted for categorical variables. In all analyses, a two-tailed P value less than 0.05 was deemed statistically significant. The statistical analysis was conducted using R 4.0.2 software (https://www.R-project.org).

3. Results

3.1. Genetic variants

A cohort of 407 children presenting with short stature, comprising 280 (68.80%) boys and 127 (31.20%) girls, underwent WES as depicted in Fig. 1. The clinical characteristics are summarized in Supplementary Table 1. The mean height SDS of the patients was −2.72 ± 0.67. The height SDS was significantly lower in patients with identified mutations than in those without (−2.95 ± 0.92 vs. −2.66 ± 0.59; $P < 0.001$). For 86 of the 407 patients (21.1%), we identified pathogenic or likely pathogenic mutations that were likely causal for short stature (Supplementary Table 2). We generated a landscape of pathogenic genetic variants in the main physiological processes associated with short stature, including both novel and reported variants (Fig. 2A). Among the 407 patients, 50 patients (61%) carried previously reported mutations, and 36 patients (41.9%) carried newly identified mutations that were consistent with the clinical phenotype of the corresponding disease (Fig. 2B).

Ultimately, 75 single-gene mutations and 11 CNVs were identified. Among the single-gene mutations, 36 were pathogenic mutations, and 39 were likely pathogenic mutations, for a total of 39 genes. These genetic variants impact a wide range of signaling pathways, including hormonal signaling, and are involved in growth plate function, such as paracrine growth factors, extracellular matrix components, and intracellular signaling molecules (Fig. 3, Supplementary Tables 3–6). Among the molecular diagnostic variants identified, 20 (26.6%) were classified as nonsense or frameshift variants, 52 (69.3%) were missense variants, and 3 (4.0%) were splicing variants. Eleven patients (12.8%) carried pathogenic or likely pathogenic large deletions known to cause growth retardation (Supplementary Table 7). In the present study, the most common gene that caused short stature was *PTPN11* (16%), and the most common type of mutation was missense mutations.

3.2. Phenotypic description of patients with genetic variants

Supplementary Tables 8–12 provide an overview of the clinical

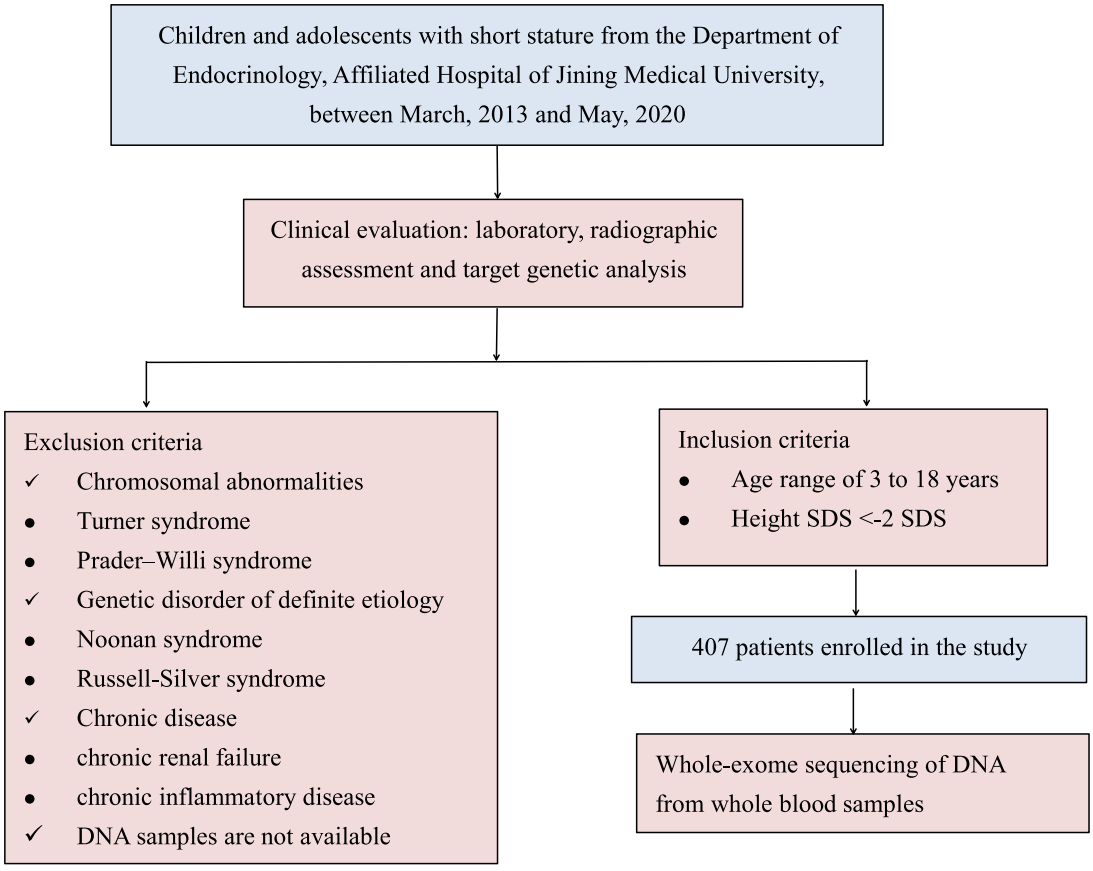

Fig. 1. Flow chart of the study population.

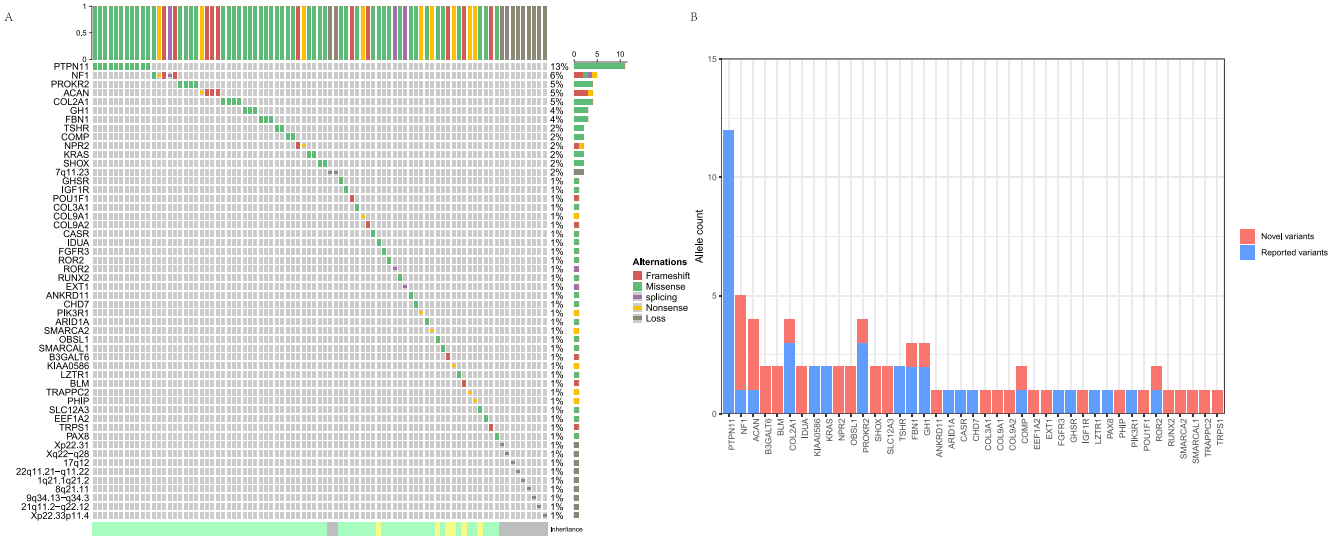

Fig. 2. Pathogenic or likely pathogenic genetic variants associated with short stature. (A): Landscape of pathogenic or likely pathogenic genetic variants in the main physiological processes associated with short stature. (B): Allele counts of pathogenic or likely pathogenic variants detected in children with short stature, including both novel and reported variants.

characteristics observed in patients with genetic variants. In patients with hormone signaling pathway defects, the predominant clinical manifestations were consistent with hormone deficiency, with growth hormone deficiency (GHD) being the most frequently observed (Supplementary Table 8). For patients with genetic defects that affected cartilage extracellular matrix and paracrine factors in the growth plate, the different gene effects manifested as bone and chondrodysplasia of varying degrees, from mild to severe (Supplementary Tables 9 and 10). Among the cohort of patients, nineteen individuals were identified to have pathogenic variants in genes associated with the RAS-MAPK pathway, specifically *PTPN11* and *NF1*. However, during the initial evaluation, their clinical presentation did not provide enough evidence to diagnose NS (OMIM #163950) or NF-1 (OMIM #162200). In addition, patients with genetic defects in fundamental cellular processes and

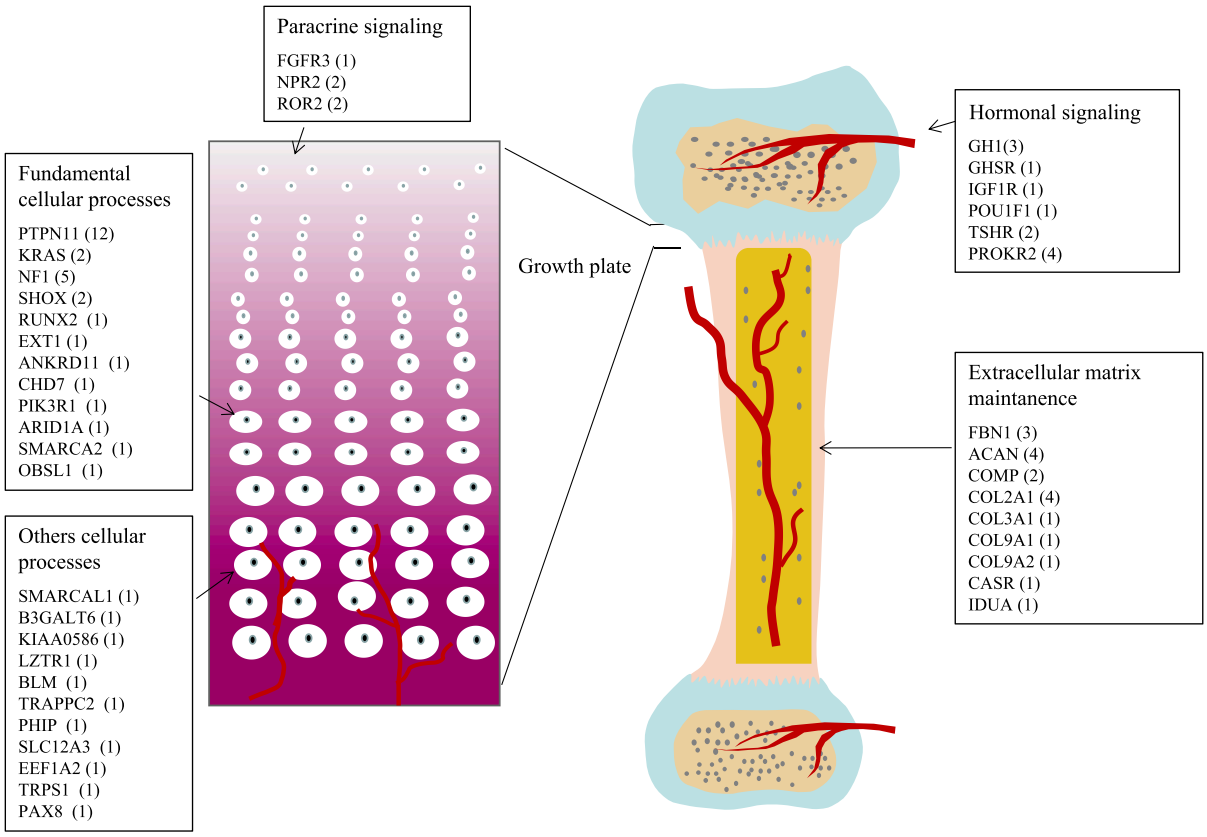

Fig. 3. Pathogenic and likely pathogenic genes in the different physiological processes associated with short stature.

other cellular processes also exhibited various clinical features associated with short stature.

Additionally, the genetic findings, including mutations in *OBSL1*, *NF1*, and *BLM*, indicate that many individuals with short stature may have syndromic diseases. Through a reassessment of certain patients, new information was obtained. Patients carrying compound heterozygous mutations in *OBSL1* exhibited features consistent with 3 M syndrome, such as short stature, large head circumference, low ear placement, wind-catching ears, long centers, thick lips, and short necks (Supplementary Table 10). Those with heterozygous mutations in the *NF1* gene presented with characteristics of NF-1, including multiple café-au-lait macules, armpit freckles, groin freckles, bilateral ear masses, and foot swelling (Supplementary Table 10). Patients with compound heterozygous mutations in the *BLM* gene, such as photosensitive erythematous skin lesions, milk coffee spots, facial elongation and narrowness, low ear placement, wind-catching ears, small chin, thick lips, high blood sugar, insulin resistance, subclinical hypothyroidism, and a small uterus, exhibited symptoms indicative of Bloom syndrome (Supplementary Table 11). Importantly, Bloom syndrome is a contraindication for rhGH treatment.

3.3. Reaction of growth to treatment with rhGH

Of 407 patients with short stature, 226 received rhGH treatment. Among 86 patients with identified pathogenic and likely pathogenic variants, 30 were treated with rhGH for various durations ranging from 0.5 to 5 years. The clinical characteristics of rhGH therapy in children with short stature are described in Supplementary Table 13. There were no significant differences in age, birth weight, sex, birth length, height SDS, body weight, IGF-1 SDS, body mass index (BMI), peak GH level or pubertal stage between the two groups of patients with and without the identified genetic mutation before receiving rhGH treatment (all $P > 0.05$).

Throughout rhGH treatment, all patients demonstrated varying degrees of catch-up growth, with the most significant increase in height typically observed during the first year of treatment (Fig. 4A). However, as the duration of rhGH treatment extended, patients without genetic mutations showed a significantly better response than those with mutations after reaching the first year mark (Fig. 4B). Patients with mutations in the hormonal signaling pathway showed the best responses to rhGH, better than both those with nonhormonal signaling gene mutations and those without identified gene mutations (Fig. 5A and B). Among all children with short stature, the patients with paracrine factor mutations had a worse response to rhGH (Fig. 5C and D).

One year after rhGH treatment onset, the absolute height SDS and the degree of improvement in height SDS showed a significant negative correlation with the age of the patients at the start of treatment ($r = -0.17$; $p = 0.032$; $r = -0.18$; $p = 0.021$; respectively; Fig. 6A and B). Patients who received treatment at a younger age had better outcomes. Further analyses showed that the relationship between age at the initiation of treatment and height SDS and the amount of improvement was significant in patients with mutations ($r = -0.17$; $p = 0.048$; $r = -0.21$; $p = 0.015$; respectively; Fig. 6C and D) but not in patients without mutations ($r = -0.19$; $p = 0.42$; $r = -0.01$; $p = 0.96$; respectively; Fig. 6C and D), suggesting that early genetic detection, early diagnosis and early treatment should be carried out to improve treatment responses. However, the number of patients without identified mutations was much smaller, which may explain the lack of a significant correlation between the age of treatment onset and height SDS or its improvement.

4. Discussion

The current study investigated the complex genetic factors contributing to short stature in a group of unrelated patients. Among the 407 children with short stature enrolled in the study, a total of 86 pathogenic

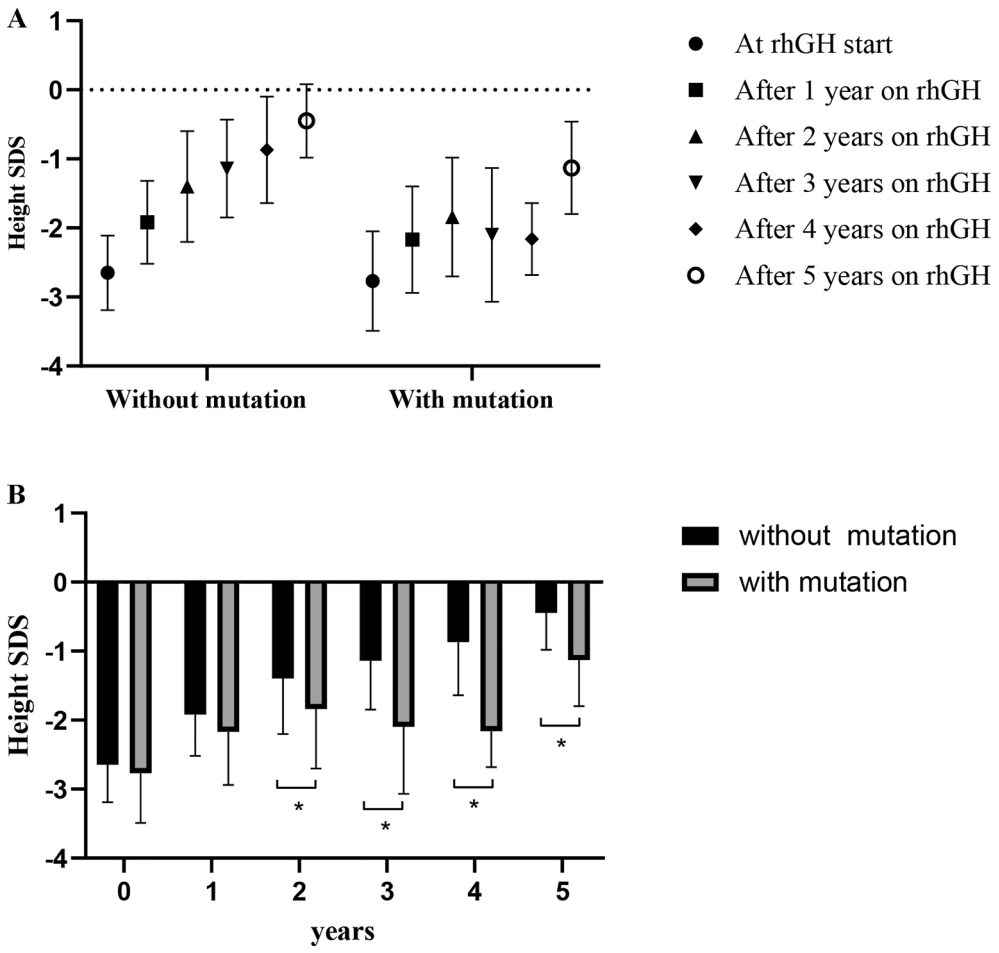

Fig. 4. Effectiveness of recombinant human growth hormone treatment in patients with and without gene mutations. (A): Improvement in height standard deviation; (B): Comparison of recombinant human growth hormone in short stature patients with and without gene mutations.

or likely pathogenic mutations associated with growth disorders were discovered. During treatment with rhGH, patients with short stature exhibited different levels of catch-up growth. Patients without identified genetic variants responded better to rhGH than those with identified potential causal mutations, except patients with mutations in the hormone signaling pathway, who showed a better response to rhGH. Patients with paracrine factor mutations had the worst response to rhGH. Furthermore, early treatment onset may lead to better clinical management and treatment options.

The significant contribution of genetic variants to short stature in this cohort should prompt reconsideration of routine mutation screening in patients with short stature without clinically significant malformations, which is currently not recommended in the management guidelines for short stature due to the rarity of presumed monogenic causes [20]. Accurately identifying the molecular causes of short stature is challenging due to its extensive etiological heterogeneity and clinical complexity. Pathogenic or likely pathogenic variants were detected in 86 (21.1%) out of 407 patients in our cohort, which is an argument for using molecular diagnosis as a factor in the consideration of treatment options. Next-generation sequencing has been extensively applied in studies on the genetic etiology of short stature, with molecular diagnosis rates ranging from 10% to 40% [21–24]. The large variation in diagnostic rates for next-generation sequencing may be due to differences in inclusion criteria, sequencing platforms, analysis processes, or diagnosis criteria.

The underlying genetic factors responsible for linear growth disorders, as identified in previous studies, are notably diverse, impacting multiple cellular pathways. Additionally, any regulatory mechanism that interferes with the process of growth plate chondrogenesis may be considered a genetic culprit in the pathogenesis of growth disorders [25]. By classifying the functional role of candidate genes in growth plates, we identified many disease-causing genes involved in fundamental cellular processes. These genes mainly encode proteins in important cellular signaling pathways, such as the RAS-MAPK pathway. Twelve patients had missense mutations in *PTPN11,* and two patients had mutations in *KRAS*, consistent with the mutation spectrum for NS (OMI #163950). The diagnosis of NS is typically established according to specific clinical criteria [26]. Initially, suspected NS is often identified by notable facial features and/or characteristic heart abnormalities. Nonetheless, the phenotype observed in the children examined in this investigation was mild, with only five individuals displaying congenital heart disease (CHD). Furthermore, certain children with unexplained short stature may display mild clinical characteristics that can be observed in multiple syndromes. However, relying solely on these features is insufficient for establishing a definite syndrome diagnosis. Consequently, syndromes with diverse clinical phenotypes, especially those with mild manifestations, may be mistakenly diagnosed as idiopathic short stature (ISS). For these cases, molecular genetic diagnosis can play a major role in reaching a definitive diagnosis and can also be factored into decision making in terms of treatment options.

The genetic variation profile of short stature identified in this study overlaps with and differs from that reported in previous studies [21–24]. Previous studies have reported that mutations in the *ACAN* gene are found in patients with short stature, especially ISS, at a frequency of 1.2% [27]. In the present study, 4 *ACAN* gene variants were identified in 407 patients with short stature, at a frequency of 1%, which was

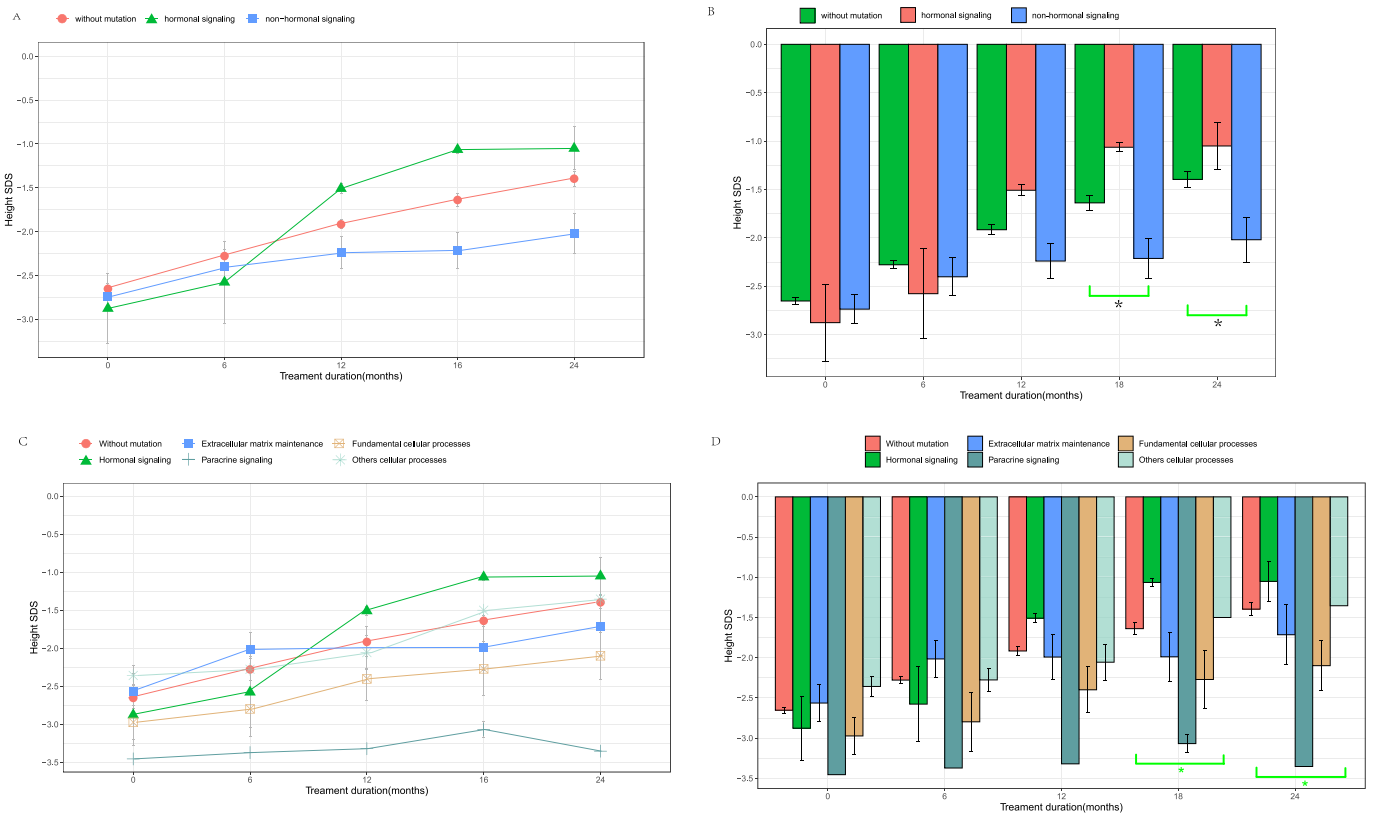

Fig. 5. Improvement and comparison of the height SDS of patients after treatment with recombinant human growth hormone. (A-B) Height SDS of patients without gene mutations and with and without hormone signaling gene mutations during recombinant human growth hormone treatment. (C-D) Height SDS of patients without gene mutations and with gene mutations in hormone signaling, extracellular matrix maintenance, paracrine signaling, fundamental cellular processes and other cellular processes during recombinant human growth hormone treatment.

consistent with previous reports. For most of the mutations identified in the study, pathophysiological effects were identified. However, the genetic variants identified as pathogenic or likely pathogenic in this study included 36 (41.9%) novel variants. The novel mutations may provide new insights into the biology of affected genes or proteins, and in addition, the genetic variation of the new loci enriches the spectrum of variations in short stature.

Genetic diagnosis plays a crucial role in confirming and drawing attention to disease manifestations that may be easily overlooked, thereby providing guidance for appropriate treatment strategies. For instance, in patients with Bloom syndrome, the use of rhGH therapy is contraindicated, and caution is advised when considering rhGH treatment in patients with NF-1. Molecular diagnostic technologies have made remarkable advancements at an astonishing pace. However, several challenges persist, including the variable expressivity of genetic mutations, the substantial phenotypic variability, the influence of environmental factors, and the complex interactions between candidate genes. Therefore, the application of genetic diagnosis requires comprehensive consideration of these complexities to ensure accurate and effective management of patients.

It is well known that rhGH replacement therapy can improve growth in childhood and adult height [28]. In the present study, patients with short stature treated with rhGH showed varying degrees of catch-up growth during rhGH treatment. Patients without genetic variants responded better to rhGH than those with genetic variants. The varying individual responses to rhGH treatment observed in patients with short stature may be partially attributed to genetic factors. A recent study explored this phenomenon, identifying genetic variations associated with GH responsiveness within a cohort of short stature patients. The study found no significant impact of common single-nucleotide polymorphisms (SNPs) on GH responsiveness, suggesting that rare genetic variants may play a greater role in influencing the rhGH response [29]. Furthermore, it has been revealed that the genes involved in the rhGH response are associated with differential gene expression and associated changes in different growth-related pathways [30].

By analyzing the efficacy of rhGH in different genetic backgrounds, we found that among all children with short stature, the patients with hormone signaling pathway mutations had the best response to rhGH, while those with paracrine factor mutations had the worst response to rhGH. The results of this study are consistent with clinical practice and with findings from previous studies showing a good response to rhGH with few adverse effects in patients with mutations in the growth hormone axis [31,32]. In addition, a study revealed that the effect of rhGH treatment in patients with pituitary dysplasia gene mutations was better than that in patients without related mutations [33]. rhGH pharmacogenomics studies have suggested that GHD has the best response to rhGH treatment, while monogenic diseases such as NS have a poor response to rhGH treatment [30]. Paracrine factors are protein molecules that play an important role in regulating growth hormone secretion and activity and thus contribute to height growth. Some defects in paracrine factor genes can impair height growth. In these conditions, rhGH treatment may not significantly increase height, as the short stature is not due to impaired secretion and activity of growth hormone but rather a result of defects in the genes relating to paracrine factors that affect bone growth. Therefore, targeted treatment methods may be necessary to address these issues and improve growth outcomes [34].

In a cohort of 407 patients with short stature, only 196 patients received rhGH treatment. We primarily consider two aspects of this phenomenon. First, prior to initiating rhGH treatment, a comprehensive assessment was conducted to exclude the potential risk of tumors. Patients who do not meet the indications for rhGH treatment, such as those at risk of tumors such as Bloom syndrome [35], are prohibited from

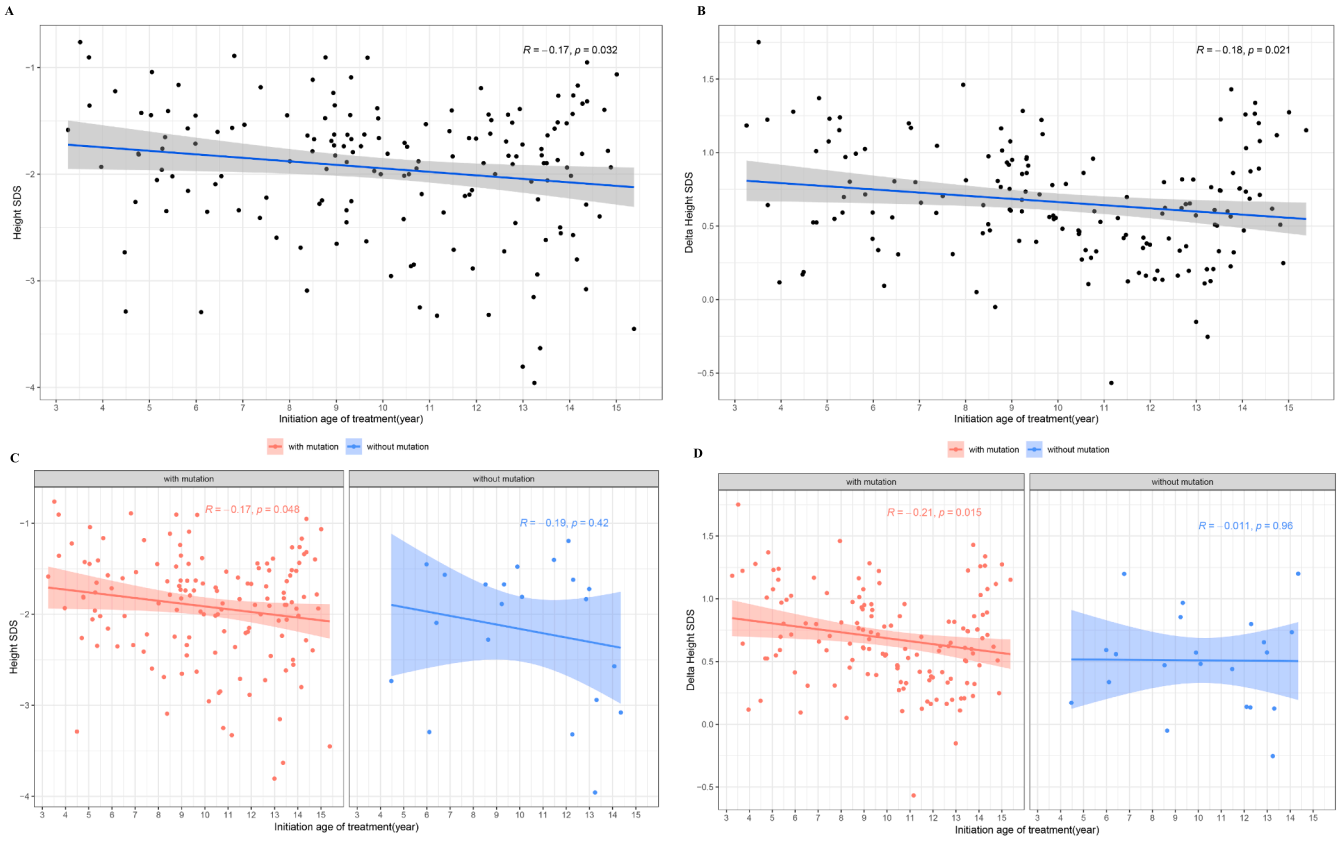

Fig. 6. The correlation between age at treatment initiation and height SDS. (A-B) Height and height SDS changes in all patients. (C-D) Height and height SDS change in patients with short stature with and without gene mutations.

receiving rhGH therapy. Similarly, rhGH treatment is not recommended for patients with single-gene tumor syndromes, such as NF-1 syndrome [36]. Second, for patients meeting the criteria for rhGH treatment, we recommend its use, but parents may have concerns about medication safety and treatment costs. Despite the proven safety of rhGH treatment [37], some parents still have concerns. Additionally, most short stature patients cannot have rhGH treatment costs reimbursed through medical insurance, placing a financial burden on their parents. Due to the high cost and long-term nature of rhGH treatment [38,39], many families opt out due to financial constraints. In the future, with the continuous advancement of medical technology and social security, it is expected that the issues faced by patients with short stature in terms of treatment cost and safety can be more comprehensively addressed, providing more patients with accessible treatment options.

The present study has several limitations. Patients with minor malformations were not limited because monogenic disease may also manifest as mild clinical features. In addition to the monogenic etiologies identified in this study, oligogenic or polygenic mechanisms may also be the cause of disease in patients who are negative for monogenic disease. Furthermore, it is difficult to reassess the underlying clinical phenotype in patients with short stature who may have specific syndromes after molecular diagnosis, but this approach will improve our understanding of rare disorders characterized by short stature and will allow for a more comprehensive evaluation of patients in future work. In addition, not all patients underwent rhGH treatment, and sample sizes when analyzing the effects of rhGH treatment in patients with different genetic backgrounds through different pathways were small. Therefore, in future studies, sample sizes should be further expanded for verification.

5. Conclusions

In conclusion, a genetic analysis of Chinese patients with short stature was conducted. We found that 86 (21.1%) of 407 patients had pathogenic or likely pathogenic mutations associated with growth disorders. After molecular diagnosis, some of the syndromic disorders characterized by short stature may be of concern for reevaluation to determine the diagnosis or to improve awareness for comprehensive evaluation in future work. We observed that patients with identified genetic mutations had significantly poorer treatment outcomes than those without identified mutations. The exception was that patients with hormone signaling pathway mutations had a better response to rhGH, while those with paracrine factor mutations had the worst response to rhGH. Our study provides evidence in favor of the use of genetic testing to identify the underlying causes of short stature and assist in making accurate diagnoses and treatment decisions. Even in the absence of physical abnormalities, short stature may be attributed to genetic abnormalities affecting hormones, paracrine factors, cartilage extracellular matrix, and essential cellular processes. Further research is needed to investigate gene-specific rhGH reactivity as an essential initial step toward improving the personalized management of short stature.

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Author contributions

Qianqian Zhao and Bo Ban conceived the study. Mei Zhang, Yanying Li, Yanhong Zhang and Qian Shao provided the clinical sample and

information. Chuanpeng Zhang and Wei Wei devised all analyses. Qianqian Zhao wrote the original manuscript. Wanling Yang and Bo Ban reviewed and edited the manuscript. All the authors have read and approved the final manuscript.

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

CRediT authorship contribution statement

Qianqian Zhao: Writing – original draft, Conceptualization. **Mei Zhang:** Investigation, Data curation. **Yanying Li:** Resources, Methodology. **Chuanpeng Zhang:** Software, Formal analysis. **Yanhong Zhang:** Resources, Methodology. **Qian Shao:** Investigation, Data curation. **Wei Wei:** Software, Formal analysis. **Wanling Yang:** Methodology, Data curation. **Bo Ban:** .

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2024.117779.

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