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GROWTH HORMONE (GH) MUTATIONS AND SEQUENCE VARIANTS IN CHILDREN WITH GROWTH HORMONE DEFICIENCY (GHD)

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Background. Isolated growth hormone deficiency (IGHD) exhibits genetic heterogeneity in both familial and sporadic cases. The *GH1* gene is a mutational hotspot among the five genes responsible for growth hormone deficiency (GHD), frequently causing short stature.

The study **aimed** to screen for two common *GH1* gene mutations prevalent in Asian populations among Sri Lankan children with biochemically confirmed GHD, and to compare sequence variations with known variants in other populations.

Methods. Genomic DNA was extracted from 10 children with confirmed GHD attending Lady Ridgeway Hospital, Colombo. PCR amplification was optimized for two primer sets targeting hotspot regions: the deletion-prone homologous flanking regions and the exon 3-intron 3-exon 4 region. Restriction fragment length polymorphism (RFLP) analysis was performed using SmaI enzyme (to detect the common 6.7 kb deletion) and NlaIII enzyme (to detect the G>A transition at the intron 3 splice donor site). Direct DNA sequencing was subsequently conducted for variant confirmation.

Results. SmaI digestion revealed heterozygous-like patterns in 3 out of 10 samples (30 %); it reflects heterozygosity or residual allele abnormalities. The remaining 7 samples (70 %) showed banding patterns identical to healthy controls. NlaIII digestion showed no mutations in any of the 10 samples (0/10, 0 %), with all patient samples displaying patterns indistinguishable from healthy DNA.

Conclusion. The two mutations commonly found in Asian populations were largely absent in this Sri Lankan cohort, suggesting that short stature in this population may result from alternative *GH1* mutations or other genetic variants, warranting comprehensive genomic screening studies.

Keywords: isolated growth hormone deficiency, *GH1* gene, polymerase chain reaction, restriction fragment length polymorphism, short stature, Sri Lankan population

INTRODUCTION

Growth hormone deficiency (GHD) is the most common pituitary hormone deficiency, defined by the complete absence or insufficient secretion of growth hormone and decreased levels of insulin-like growth factor-1 (IGF-1). GHD occurs as isolated growth hormone deficiency (IGHD), where only GH is deficient, or combined growth hormone deficiency, involving deficiencies in multiple pituitary hormones including ACTH, TSH, and gonadotropins (Smyczyńska *et al*., 2022). IGHD occurs in 1 in 4000–10000 births (Marikar *et al*., 2006; Ye *et al*., 2008), with 5–30 % of cases attributed to genetic etiology, particularly in consanguineous families, while 40–60 % show anatomical anomalies of the hypothalamic-pituitary region (Lewiński *et al*., 2021).

GHD results from defects in five main genes in the GHRH-GH axis: growth hormone gene (*GH1*), growth hormone-releasing hormone receptor gene (GHRHR), and growth hormone receptor gene. Growth hormone is secreted in a pulsatile manner with peak levels at night, and acts primarily through GH receptors in the liver and other target organs to stimulate IGF-1 release (Goldenberg *et al*., 2021; Devesa & Devesa, 2023; Mastromauro *et al*., 2023). Growth hormone exerts endocrine, paracrine, and autocrine actions, regulating growth through both IGF-1-dependent and independent pathways (Birzniece and Ho, 2021; Chesnokova and Melmed, 2023).

The *GH1* gene is a major mutational hotspot causing short stature. IGHD type IA, the most severe form, is characterized by complete *GH1* deletion (both alleles), growth retardation, characteristic facial appearance, and infantile hypoglycemia (Arai-Ichinoi *et al*., 2021). Deletion sizes vary by ethnicity, ranging from 6.7 kb to 45 kb (Joshi *et al*., 2021). Common mutations include the G>A transition at the intron 3 splice donor site, a mutational hotspot due to spontaneous deamination of methylated cytosine in CpG dinucleotides (Bioletto *et al*., 2024; Tamhankar *et al*., 2022; Thompson *et al*., 2024).

This study aims to detect mutations commonly found in Asian populations among Sri Lankan children with biochemically confirmed short stature, and to compare sequence variations with known variants in other populations. Early genetic diagnosis enables precise screening in neonatal or early childhood stages and facilitates alternative treatments such as IGF-1 therapy for patients who develop anti-GH antibodies (Bailes and Soloviev, 2021), opening new pathways for hormone research and gene therapy applications. Therefore, studying GH-associated mutations for early diagnosis and developing successful treatments will open new pathways for hormone research. The objectives are to identify mutations in the growth hormone gene in children with growth hormone deficiency and to compare sequence variations in the growth hormone gene in the Sri Lankan population with currently identified variants in other populations.

MATERIALS AND METHODOLOGY

Subject. Children with short stature who have been biochemically confirmed to have growth hormone deficiency who visit the clinic (Lady Ridgeway Hospital – Professorial Unit) for scheduled reviews comprised the study group. The records of former patients who attended Lady Ridgeway Clinic, Borella, Sri Lanka, were reviewed for evaluation

of short stature. Eligible subjects were traced using clinical registries and invited to participate in clinical visits. Subjects eligible for participation fulfilled the following inclusion criteria: short stature defined as a height over two standard deviations below the population mean. Subjects with endocrine or metabolic disorders or with primary or secondary growth disorders were excluded.

Sample collection. A single blood sample of 2.5 mL was collected under aseptic conditions for genetic analysis from the patients who were biochemically identified as growth hormone-deficient children in Lady Ridgeway Hospital, Colombo. The blood samples were collected into the heparin-coated tubes, labelled (GH-001 etc) and stored at 70 °C to be used for the DNA extraction. The total number of samples in this study was 10.

Polymerase chain reaction. PCR amplification of genomic DNA was performed with primers designed to amplify a 1905-bp portion of the region coding for the extracellular domain of GH. The whole sequence of the growth hormone gene and the sequence of the intron 3 regions, the region where numerous mutations are present among the Asian population, were obtained from the NCBI website. One primer set for deletion was designed to the homologous region outside the *GH1* gene according to Li (Li *et al*., 2025). To confirm a deletion, the *GH1* gene was amplified with primers *GH1*de-F1 (GGATCCAGCCTCAAAGAGCTTAC) and *GH1*de-R1 (AGGTAACGAGTTCCGAGAC CCTTAG) that specifically amplify *GH1* and do not recognize homologous genes in the cluster. The PCR conditions were 2 min at 94 °C; 30 cycles of 45 s at 94 °C, 15 s at 65 °C, 3 min at 72 °C; and ending with 3 min at 72 °C. The PCR products were digested with SmaI. Amplification products were visualized on 2 % agarose gel and stained with ethidium bromide. The primer set for mutation was designed to cover the whole gene region including exon 3, intron 3, and exon 4 using the NCBI/Primer BLAST software programme. The primers were synthesised by the Integrated DNA Technologies, USA (Boston). To characterize the size of *GH1* gene deletions, two highly homologous regions flanking the *GH1* gene were amplified using primers *GH1mu F1* (AATGGGAGCTGGTCTCCAGCG) and *GH1*mu-R1 (GGGGCTC CAGGATTGGGGAC). The PCR conditions were 10 min at 95 °C; 1 min at 85 °C; 30 cycles of 30 s at 94 °C, 30 s at 60 °C, 5 min at 72 °C; and ending with 8 min at 72°C. The PCR products were digested with NlaIII. Amplification products were visualized on 2 % agarose gel and stained with ethidium bromide.

Sequencing. DNA sequencing is a very important method in every field especially in the diagnosis of diseases. There are several types of techniques to obtain the sequence of the genetic material. PCR products that show abnormal banding patterns are subjected to direct sequence using a MegaBACE 1000 DNA sequencer. Resulting DNA sequences are subjected to bioinformatics analysis to identify mutations and sequence variants. Identified mutations and sequence variants are confirmed by analysis of a second aliquot of DNA stored. Sequences obtained are subjected to bioinformatics analysis. Mutations and sequence variants confirmed in the studied sample, as well as their frequencies, are reported.

Compliance with ethical standards and the informed consent. This study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki. The study received ethical approval from the Ethics Review Committee of the University of Colombo, Colombo, Sri Lanka (Approval No: ERC/UOC/GH&GHRH/2023_011, dated June 21, 2023). Written informed consent was obtained from the parents or legal

guardians of all participating children prior to sample collection and genetic analysis. The purpose, procedures, potential risks, and benefits of the study were fully explained to the parents/guardians, and their right to withdraw from the study at any time was emphasized.

Confidentiality. All patient data and biological samples were coded and handled confidentially. Personal identifying information was kept separate from research data to ensure participant anonymity. Only authorized research personnel had access to the study data.

RESULTS

Quantification of the extracted blood DNA. Gel electrophoresis was performed to assess the quality and quantity of the extracted DNA samples. The extracted DNA was of good quality, with no RNA contamination detected. These samples showed high concentrations – more than 200 ng/μL; therefore, a 1:10 dilution series was prepared, and gel electrophoresis was carried out to obtain a more accurate estimation of the samples' concentration.

The concentrations of the diluted DNA samples of the patients were estimated by comparing the intensities of bands with the standards of known concentration. The concentration of the sample DNA of the patients was compared and the concentrations varied from 25 ng/μL to 150 ng/μL.

Optimisation of the PCR parameters for the mutation primer set. To identify the optimum annealing temperature for the mutation primer set, a temperature gradient was carried out with different annealing temperatures. The products obtained were run in a 0.8 % agarose gel and visualised under the UV transilluminator. The most intense band was present in the third well and that is with the annealing temperature of 60.9 °C, therefore it was chosen as the optimum annealing temperature of the primers to the DNA template.

With the use of the optimum annealing temperature, an Mg^{2+} concentration gradient was carried out to get the optimum Mg^{2+} concentration, which gives a high yield of the product. The product was visualised under the UV transilluminator. According to the concentration gradient carried out with different Mg^{2+} concentrations, the optimum concentration of Mg^{2+} that gave a high yield of the product was of 3mM Mg^{2+} concentration.

The primer concentration gradient was carried out to obtain a high yield of the product with smaller amounts of the primer dimmers. The gel electrophoresis was carried out and visualised under the UV transilluminator and the results were obtained. The optimal primer concentration, which gave the least primer dimmers and a high yield of the product, was present in the 0.25 μM primer concentration.

Estimation of the size of the PCR product. Two microlitres of the PCR product obtained under optimum conditions, mixed with loading dye, were run with 1 μL of the 100bp ladder mixed with 1 μL of loading dye and 1 μL of water. The gel electrophoresis was carried out for about 1 hour at 50V till the ladder got separated as shown in **Fig. 1*A***.

The size of the PCR product was compared with the bands of the 100bp ladder and was estimated to be approximately 450bp (the precise size of the product is 465bp).

PCR with the mutation primer set. After optimising all the parameters to obtain a high yield of the product, the PCR reaction was carried out with different samples.

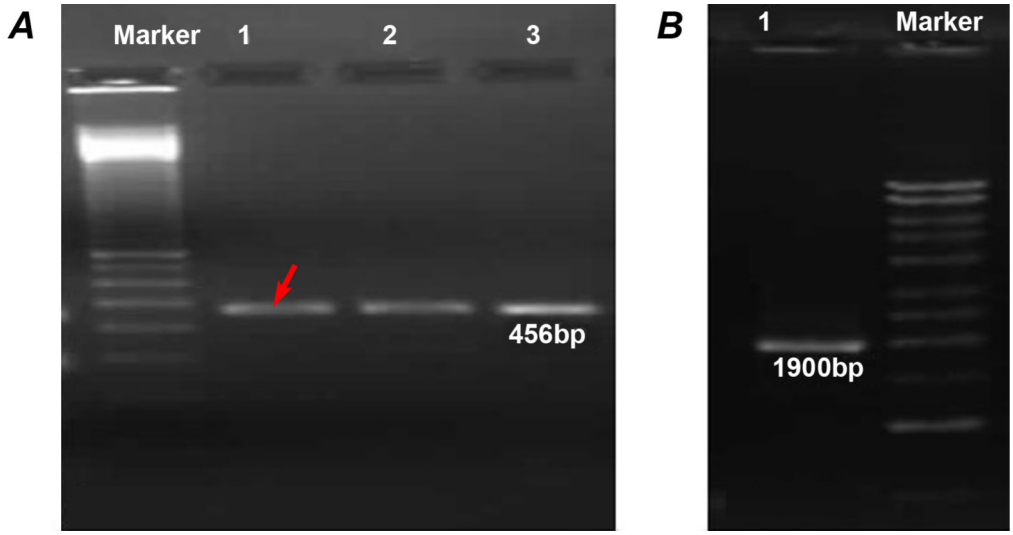

Fig. 1. ***A*** – estimation of the size of the PCR product carried out with the mutation primer set. Lane 1 100bp ladder 1,2,3 is PCR products obtained from the mutation primer set; ***B*** – estimation of the fragment size of the PCR product obtained from the deletion primer set. The PCR product and the 1000bp ladder are loaded respectively

Optimisation of PCR parameters for the deletion primer set. The optimum annealing temperature for the deletion primer set was determined by performing PCR reactions at different temperatures ranging between the melting temperatures of the two primers. The optimum annealing temperature for the deletion primer set was 59.9 °C. Subsequently, a Mg^{2+} gradient was carried out with different concentrations of Mg^{2+} and the optimum concentration was determined by gel electrophoresis. According to the gel image, the most favourable Mg^{2+} concentration was 1.5 mM.

Optimisation of primer concentration. The PCR reaction was carried out with different primer concentrations to detect the best primer concentration with a reduced amount of primer dimmers and a high yield of the product. The product with less amounts of primer dimmers was shown in the primer concentrations of 0.4 µM, accordingly, it was chosen as the optimised primer concentration.

Optimisation of DNA concentration. To detect the exact amount of the template DNA needed for the PCR reaction, a concentration gradient of the healthy DNA samples and the patient DNA samples was carried out. The best possible concentration of DNA used in the PCR reaction was of 150 ng.

Estimation of the size of the PCR product. The PCR reaction was carried out with the optimum PCR conditions; 2 µL of the PCR product along with the loading dye was run in an agarose gel with t1 µL of the 1000bp ladder mixed with water and loading dye. The gel electrophoresis was carried out and visualised under the UV transilluminator as shown in **Fig. 1*B***. The size of the PCR product is approximately 1900bp. The PCR reaction for the patients' DNA samples was carried out with the optimised PCR parameters.

Quantification of the purified PCR products. The PCR products were then purified using the ethanol purification method prior to use in the RFLP. The samples were subjected to gel electrophoresis with the standards of known concentrations and visualised under the UV transilluminator. The concentrations of each sample obtained from both primers were estimated by comparing the intensities of the bands. The concentration of the purified PCR samples obtained from the mutation primer set was approximately 60 ng/μL. The concentration of the purified PCR samples obtained from the deletion primer set was 60 ng/μL – 150 ng/μL.

Visualisation and analysis of RFLP products

RFLP analysis with the SmaI enzyme to detect the 6.7 kb deletion. After the RFLP reaction was carried out with the SmaI restriction enzyme, 15 μL of the RFLP product was mixed with 5–7 μL of the loading dye and was subjected to gel electrophoresis in a 1 % agarose gel. The banding pattern was as shown in **Fig. 2*A***.

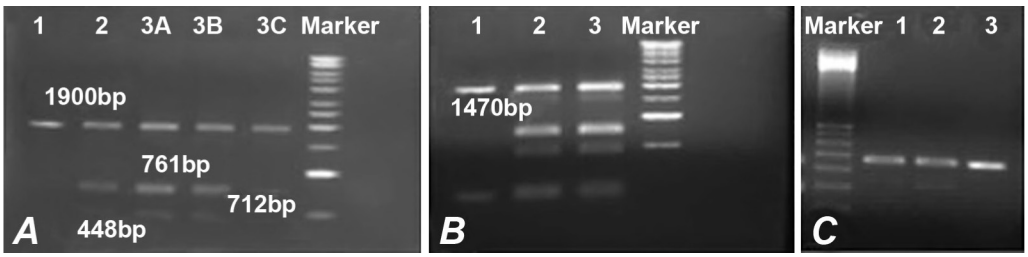

Fig. 2. **A** – visualisation of the SmaI digested RFLP products. The samples were loaded as 1) the undigested PCR product, 2) healthy DNA sample, 3) patients' samples, 4) ladder; ***B*** – RFLP analysis with the SmaI enzyme for patients' samples showing a heterozygous condition. 1) The PCR product without the restriction enzyme 2), 3) PCR products of the patients' samples with the enzyme; ***C*** – The RFLP analysis carried out with NlaIII enzyme. The samples were loaded as 1) the ladder, 2) digested patient sample, 3) digested healthy sample, 4) sample without the restriction enzyme

The RFLP fragment sizes were compared with the ladder and the sizes of the fragments were estimated. The PCR sample without the restriction enzyme was not digested. The samples treated with the restriction enzyme were digested into three fragments approximately with the sizes as the fragment without being digested was 1900bp, one prominent band around the 750bp region which contains 761bp, and 712bp bands, which did not get separated further, as well as a 448bp band.

The RFLP was carried out for other samples as shown in **Fig. 2*B***, two samples showed four bands of 1900bp, 1470bp, 761bp, and 712bp, which did not get separated, and a 448bp band.

These samples with four bands represent the heterozygous condition, containing an additional band of 1470bp, which is not observed in the healthy sample. Further analysis was carried out with another set of samples; the results were the same. These samples showed the digestion and showed three sizes of bands of 1900bp, 761bp, and 712bp, which did not get separated, as well as a 448bp band, the same as that of the healthy sample.

RFLP analysis with NlaIII enzyme to detect the intron 3 G>A transversion. After the RFLP reaction was carried out with the NlaIII restriction enzyme, 15 μL of the

RFLP product was mixed with 5–7 μL of the loading dye and was subjected to gel electrophoresis in a 1 % agarose gel. A 100bp ladder was loaded to compare the size of the digested samples. The banding pattern was as shown in **Fig. 2*C***.

The RFLP products of the healthy sample or the patient sample were not digested. The healthy sample does not contain a restriction site, accordingly, it was not cut. The RFLP was carried out for several other samples including a diluted genomic DNA. The genomic DNA was digested confirming that the enzyme is active in such conditions. Accordingly, the RFLP analysis was carried out further to observe whether the other samples contain a restriction site for NlaIII. Those samples also did not get digested like the healthy sample, as shown in **Fig. 2*C***. The healthy sample does not have any cutting site for the NlaIII enzyme; accordingly, it did not show any bands after digestion and the patients' samples were also the same as the healthy sample.

GFX column purification for the sequencing

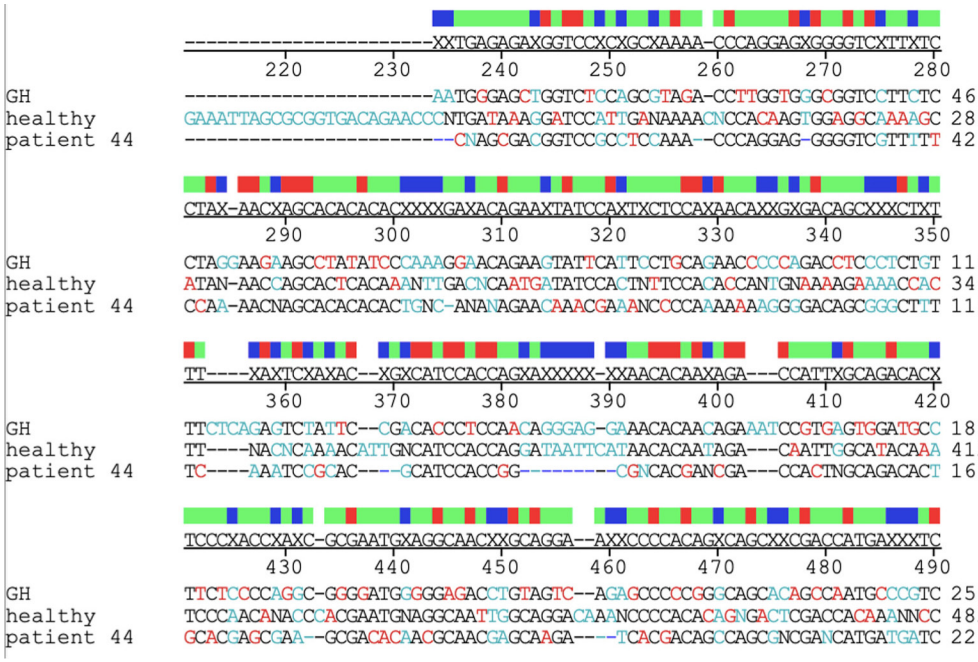

Fig. 3. Multiple Sequence Alignment of GH Healthy versus Patient 44

In this study, genomic DNA was extracted from samples obtained from both healthy individuals and patients for the analysis of the growth hormone (*GH*) gene. The GFX column purification method (GE Healthcare) was used to isolate and purify high-quality DNA, ensuring suitability for downstream molecular applications. This process involved cell lysis, binding of DNA to a silica membrane under optimized high-salt conditions, and a series of wash steps to remove proteins, salts, and other impurities. The DNA was eluted in nuclease-free water and quantified using spectrophotometry. The purified DNA was then used for PCR amplification and sequencing of the *GH* gene, and the resulting sequences were subjected to multiple sequence alignment (MSA) to identify conserved regions and detect genetic variations among the healthy and patient samples (**Fig. 3**).

DISCUSSION

The growth hormone gene is one of the target genes present in the GH-IGF axis which is responsible for the GH regulation (Caputo *et al*., 2021). Up to date it has been identified that the defects in these genes are responsible for the GHD, which is classified under the genetic aetiology (Fourneaux *et al*., 2022). The incidence of GHD has been estimated to be 1:4000 and 1:10000 live births. From 5 % to 30 % of the cases of the isolated GHD involve a genetic aetiology, and 40–60 % shows anatomical anomalies of the hypothalamic pituitary region with still unknown genetic defects (Sav *et al*., 2025). The aetiology of any case of GHD is still unknown accordingly, under the category of idiopathic GHD (Melmed *et al*., 2022). Mutations under this category may appear in patients either due to carrier parents, parents with consanguineous marriages, or sometimes due to unknown reasons. The timely diagnosis and management of GHD enables children with short stature to achieve a normal height. This will prevent their antisocial behaviour, resulting from the short stature, and will lead to a better adulthood.

The diagnosis of GHD is complicated and involves clinical tests, molecular biology tests and biochemical assays. The most sensitive diagnostic test – the imaging technique – is a very expensive technique and not available in many countries (Yuen *et al*., 2023). With the advancement of investigations, the biochemical assay methods and genetic testing play a major role in diagnosis. But the main drawback in the biochemical tests is the differences in the cut-off levels with time and the overlapping results between the children with GHD and normal children, and the cut-off levels should be defined in each assay (Zaffanello *et al*., 2024). Out of these techniques, molecular tests which diagnose the mutations present in the genes responsible for growth become the priority.

Numerous mutations in the growth hormone gene have been identified worldwide. Even though many mutations have been reported, these mutations differ with the ethnicity. Sri Lankan population may show the identical mutations to those of another country basically due to a mutation present among the Asian population or it may even be due to a new mutation unique to our population. Therefore, the genetic testing to detect the mutation plays a vital role. Accordingly, to detect the specific mutations among the Sri Lankan population, blood was collected from the biochemically identified children with GHD. According to the mutation map, the most common mutation detected among the Asian population was the deletion in the growth hormone gene and the mutation in the intron 3 region. This study was mainly based on these two mutations.

The PCR reaction was carried out by using primers designed to amplify the mutational hot spots. As the PCR protocol is specific to each primer set, the PCR reaction requires optimisation to get a high yield of the product. As the PCR is a very sensitive method, precautions have been taken to prevent contaminations throughout the process. A temperature gradient was carried out with both sets of primers to get the optimum annealing temperature. The gradient was taken around the melting temperatures of the two primers. The optimum annealing temperature for the mutation primer set was 60.9 °C and the deletion primer set showed the optimum annealing temperature of 59.9 °C (60 °C).

Magnesium acts as a cofactor for the polymerase enzyme as it is a magnesium-dependent enzyme. Determination of the optimum concentration to use is critical to the success of the PCR reaction as high concentrations of Mg^{2+} ions give rise to undesired products and low concentrations form low yield of the product. The optimum

Mg^{2+} concentration for the mutation primer set was 3mM, while for the deletion primer set it was 1.5 mM. The primer concentration was also optimised as the formation of primer dimmers reduced the yield of the product. The lower primer concentrations showed less yield of the product. Accordingly, after comparing the amount of the product and the primer dimmers, the optimum mutation primer concentration was estimated to be 0.25 µM, while for the deletion primer set the best primer concentration was 0.4 µM.

Further optimisation of the DNA concentration was carried out with the deletion primer set to optimise the amount of template DNA used in the reaction. The template quality and quantity also affect the amplification depending on the DNA quality and quantity. Most of the reagents (salts, guanidine, proteases, organic solvents and SDS) used in the extraction and purification of nucleic acids act as inhibitors of DNA polymerases. The quantity of the template used in the reaction is a prime factor in the PCR reaction. Reactions with too little DNA give low yield, whereas too much DNA may give nonspecific amplifications. It is recommended to start with $>10^4$ copies of the target sequence to obtain a signal in 25–30 cycles and try to maintain the final DNA concentration of the reaction ≤10 ng/µL.

Apart from optimising the above requirements, the PCR enhancers and additives can be added for a successful PCR method. Yield of the PCR product can be increased by adding PCR-enhancing agents which utilise different mechanisms to enhance the PCR productivity. DMSO, formamide, the general stabilizing agents like BSA (0.1mg/mL, gelatine (0.1–1.0 %) and non-ionic detergents (0–0.5 %) can solve the amplification failures. They increase the stability of the DNA polymerase and decrease the loss of reagents via adsorption to tube walls. Non-ionic detergents, such as Tween®-20, NP-40 and Triton® X-100 can be used to overcome inhibitory effects of trace amounts of strong ionic detergents, such as 0.01 % SDS. PCR reaction was carried out with these optimised concentrations, but further optimisation with the dNTP and Taq polymerases was not carried out as the PCR reaction gave a high yield of the product minimizing the waste and cost of a reaction.

The PCR products obtained with the optimised protocol, the products obtained from the mutation primer set and the deletion primer set were run in an agarose gel along with a 100bp ladder and a 1000bp ladder, respectively. The size of the product of the mutation primer set was approximately 450bp, confirming its exact size of 465bp. The size of the PCR product of the deletion primer set was 1900bp. The PCR products were then purified by the ethanol precipitation method. The PCR product purification is carried out to remove all the unnecessary components, such as the excess buffer, salt, enzymes present in the product and to obtain a pure product for further enzymatic manipulations. The pure DNA is a must for the RFLP analysis as the impurities act as inhibitors of the digestion.

Initially precipitation was done only with sodium acetate and absolute ethanol, but the purification yields a low amount of the product. Adjustments were made to enhance the precipitation by reducing the loss of the PCR product. In addition to the sodium acetate and the absolute ethanol, 2 µL of 10 mg/mL glycogen was added to the mixture. It enhanced the purification and precipitation of the PCR products. The concentration of the purified products is important in RFLP. The PCR products of the mutation primer set needed about 350–450 ng of the product. The PCR products of the deletion primer set needed at least about 500 ng of PCR product. Accordingly, the PCR reaction was repeated for several samples to obtain a high yield of the product. Even though

the purification was carried out, the primer dimmers were present in some samples, they would not interfere with the RFLP as the primer dimmers are about 50bp and the expected fragment sizes of the digested products are not less than 100bp. Accordingly, the primer dimmers can be distinguished from the fragments, and will not mask the fragments.

The RFLP protocol was optimised to provide the optimum conditions for the restriction enzyme activity. Different incubation time, different concentrations of the product were used in order to get the most suitable values for the digestion. The incubation temperature was checked from time to time. RFLP was carried out with 10 samples of each PCR product and the fragment sizes were compared with the healthy sample. The RFLP was carried out with the SmaI restriction enzyme according to the Vnencak–Jones method. The PCR products of the healthy individuals (1900bp and 1921bp in healthy individuals) contain two SmaI sites in the 1921bp product, but not in the 1900bp product. There is only one restriction site present in the patients with the expected mutation (1918bp).

Accordingly, RFLP analysis was carried out in the IBMBB lab, Colombo, Sri Lanka. The healthy individuals gave a 1900bp fragment, 761bp, and 712bp fragments, which did not get separated, and a 448bp fragment. The patients' samples also gave the same banding pattern as shown in **Fig. 2**. Three out of ten samples showed a heterozygous condition, as shown in **Fig. 2**. These samples produced a distinctive pattern following the SmaI digestion, yielding fragments of 1900bp, 1470bp, 761bp and 712bp, which did not get separated, as well as a 448bp fragment, confirming the heterozygous condition. The 761bp and 712bp bands did not get separated in the 1 % agarose gel but it did not disturb the results as the healthy individuals as well as the heterozygous individuals both contain these bands, and the homozygous individuals do not show these bands; accordingly, the separation is not needed. Due to the time constraints, the RFLP could not be repeated, and these results could not be confirmed again.

Secondly, the RFLP analysis was carried out with the NlaIII enzyme to detect the G>A transversion in the 1st nucleotide of the invariant GT dinucleotide of the 5′ donor site of intron 3 region. This mutation was reported to be present in many ethnic groups (Chalazan *et al*., 2021). This mutation region is identified as a mutational hot spot due to the presence of the CpG dinucleotide which has an increased mutation rate (Tomkova *et al.,* 2024). Accordingly, this region was selected to study. From numerous mutations in this region a particular mutation G>A transition was selected as the basis of RFLP analysis. Healthy individuals do not possess a site for the NlaIII enzyme; however, patients with the expected mutation create a site for the NlaIII enzyme thus yielding (Viswanathan *et al*., 2023) two fragments of 173bp and 292bp. Nevertheless, the 10 studied patients in the Sri Lankan population with short stature did not show any significant digestion in RFLP with NlaIII enzyme. Accordingly, this polymorphism may not account for the short stature in the Sri Lankan population. According to the pedigree details, the study group of the Sri Lankan population does not have any genetic background. The parents of each family are of normal height and the family members of these families were of normal height. But as these types of genetic mutations can also be due to the carrier parents and may be due to any other sporadic cases (*de novo* mutations), further studies should be done to detect other types of mutations.

According to the literature, 5–30 % of the cases have been identified to have the genetic aetiology and many cases are still due to unknown reasons. Out of these, there

are numerous defects found in the *GH1* gene that is responsible for short stature. Due to the time constraint, the study was based on the most widespread mutations present among the Asian populations and addressed only two mutations. However, the Sri Lankan population did not show the expected mutations, and the short stature is due to another mutation. As the SmaI digestion with 3 patients showed the heterozygous condition (further studies must confirm this condition), the patients must include another abnormality in the remaining allele causing the growth retardation. These findings are compatible with heterozygosity and need confirmation using higher-resolution tests (e.g., MLPA/copy-number testing and/or full *GH1* sequencing) to determine the exact deletion and its clinical relevance. It is important to confirm these results as it could provide for a prenatal diagnosis, since the deletion in one allele could be a risk factor in pregnancies for their future children. In addition, homozygous patients with the 6.7 kb deletion can be identified by assessing anti-GH antibody formation, as these individuals produce antibodies in response to GH therapy. According to the literature, many reports have shown differences in both the location and size of the deletion, ranging from 6.7 kb to 7.6 kb.

Accordingly, further studies are warranted to investigate deletions in the Sri Lankan population and to clarify the molecular basis of these deletions. In addition, as the Sri Lankan population has not shown the G>A transition so far, further studies are needed to detect other possible mutations in this region that may account for growth retardation. As previously noted, the amplified region represents a mutation hotspot; therefore, short stature in the Sri Lankan population may be due to other mutations within this hotspot. Further studies can be conducted using the same primer set, as the primers were designed to amplify the exon 3- exon 4 regions, where many mutations occur. Sequencing of this region would provide a better understanding of such mutations. Due to time constraints, sequencing could not be performed for all samples; therefore, future studies should focus on this region. Additionally, pedigree analysis of patients in the Sri Lankan population would be useful for identifying mutations among populations.

Additionally, future directions can be taken to detect the mutations by using single standard confirmation polymorphisms. The SSCP method detects conformational differences caused by SNP in the gene and identifies them by the differences in the electrophoretic mobility in a polyacrylamide gel. This method can be modified into a recent new technique, dideoxy fingerprinting, in which dideoxy sequencing reaction is carried out with a single dideoxy nucleotide on PCR products to generate DNA of different lengths. This method shows the location of the SNP and increases the accuracy of the test (Halmos *et al*., 2023).

CONCLUSION

This study investigated the presence of two common *GH1* gene mutations associated with isolated growth hormone deficiency in the Sri Lankan pediatric population. Through optimized PCR amplification, RFLP analysis, and direct DNA sequencing, we screened for known hotspot mutations frequently observed in Asian populations. While the majority of samples showed no evidence of the classic 6.7 kb deletion or the G>A transition at the intron 3 splice donor site, three samples demonstrated heterozygous patterns in the deletion screening, suggesting possible compound heterozygosity or other allelic abnormalities that warrant further investigation. The absence of these specific mutations in most Sri Lankan children with growth hormone deficiency indicates that

the genetic etiology of short stature in this population may involve alternative molecular mechanisms. These could include other mutations within the *GH1* gene, variants in the remaining four genes associated with IGHD (GHRHR, GH-releasing hormone receptor, PROP1, POU1F1, and SOX3), or yet uncharacterized genetic factors unique to the Sri Lankan population. These findings underscore the importance of population-specific genetic screening and highlight the need for comprehensive genomic approaches in future studies. Whole exome sequencing or targeted next-generation sequencing panels encompassing all known GHD-associated genes would provide a more complete understanding of the genetic landscape of growth hormone deficiency in Sri Lanka. Such investigations will not only improve diagnostic accuracy but also facilitate personalized treatment strategies and genetic counseling for affected families in this population.

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COMPLIANCE WITH ETHICAL STANDARTS

Conflictof interest: the authors declare that the study was conducted in the absence of any commercial or financialrelationship that could be construed as a potential conflict of interest

Animal rights: this article does not include animal studies.

Human rights: all studies were conducted in accordance with the Declaration of Helsinki guidelines. The study received ethical approval from the Ethics Review Committee of the University of Colombo, Colombo, Sri Lanka (Approval No: ERC/UOC/GH&GHRH/2023_011, dated June 21, 2023).

AUTHOR'S CONTRIBUTIONS

Conceptualization, [U.G.I.U.K.]; methodology, [F.M.M.T.M.]; validation, [F.M.M.T.M.]; formal analysis, [U.G.I.U.K.]; investigation, [U.G.I.U.K.]; resources, [F.M.M.T.M.]; data curation, [U.G.I.U.K.]; writing – original draft preparation, [U.G.I.U.K.]; writing – review and editing, [F.M.M.T.M.]; visualization, [U.G.I.U.K.]; supervision, [U.G.I.U.K.]; project administration, [F.M.M.T.M.]; funding acquisition, [F.M.M.T.M.].

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SUPPLEMENTARY MATERIALS

PCR Optimization

Optimisation of the Mg^{2+} concentration. The PCR reaction was carried out using the optimised annealing temperature with different $MgCl_2$ concentrations. The $MgCl_2$ concentrations ranged from 2 mM, 3 mM, 4 mM, 5 mM, to 6 mM, while all other parameters were kept constant. The negative PCR reaction was carried out with 2 µL of distilled water and 3 µM $MgCl_2$ concentration. The PCR products were visualised as above.

Optimisation of the Primer concentration. Using the optimised annealing temperature and the $MgCl_2$ concentration, the PCR reaction was carried out with different primer concentrations which varied from 0.1 µM, 0.15 µM, 0.2 µM, 0.25 µM, 0.3 µM whereas keeping other parameters constant. The negative reaction was prepared with 2 µL of distilled water and 0.1 µM primer concentration.

Optimisation of the concentration gradient. A PCR reaction with different concentrations was carried out for the healthy individuals and for the samples. The concentrations ranged from 50 ng, 100 ng, to 200 ng, while the other conditions was kept steady. The negative reaction was carried out with 2 µL of distilled water as an alternative to that of the different concentrations of the DNA used.

Blood DNA extraction. Genomic DNA was isolated from peripheral-blood samples obtained from the 10 confirmed GH deficiency children, according to standard techniques. A 3mL blood sample from the heparinised tubes was transferred to a 15 mL falcon tube. Then, 9 mL of cold lysis buffer was added and mixed gently to avoid frothing. The mixture was kept on crushed ice for 15 min and centrifuged at 3000 rpm for 20 min at 4 °C. The supernatant was discarded, and the pallet was resuspended in 9 mL of cold lysis buffer and mixed well by vortexing. The mixture was kept on ice for 15 min and centrifuged at 3000 rpm for 20 min at 4 °C. The supernatant was discarded, and the pallet was resuspended in 3 mL of cold SE buffer and mixed well by vortexing. It was then centrifuged at 3000 rpm for 15 min at 4 °C. The supernatant was discarded, and the pallet was resuspended in 3 mL of cold SE buffer by brief vortexing. The mixture was centrifuged at 3000 rpm for 15 min at 4 °C. The pallet was resuspended in 1.5 mL of SE buffer by brief vortexing. Then, 150 µL of 10 % SDS was added and mixed gently, after which 7.5 µL of proteinase K was added to the mixture and mixed well. The mixture was incubated overnight. A volume of 0.6 mL of saturated NaCl solution was added to the clear solution and was shaken vigorously for 15 seconds. The suspension was centrifuged at 3000 rpm for 15 min at 20 °C. The supernatant was transferred into a sterile falcon tube without disturbing the pallet. Genomic DNA was precipitated by adding two volumes of ice-cold absolute ethanol and mixing gently by inverting. DNA was precipitated and spool out by using a pipette and kept on the lid of the Eppendorf containing 70 % ethanol or transfered straight to 70 % ethanol. The DNA was washed by giving a small spin at 12000 rpm for 3 min. The ethanol was removed by using a pipette and the remainder was removed by air drying method. A volume of 100–250 µL of TE was added according to the size of the pallet. It was stored at 4 °C and then transferred to -20 °C. Finally, the quality and the quantity of DNA were detected by gel electrophoresis. The extracted DNA samples were run in an agarose gel to find the genomic DNA availability.

PCR product purification. The PCR product is purified prior to use for the upstream applications as RFLP in order to remove the salts, buffers and any other contaminant which can interfere with the further applications. A volume of 2 µL of 3 M sodium acetate of pH 5.2 and 40 µL of absolute ethanol was added to 15–20 µL of the PCR product in a 1.5 mL Eppendorf tube and mixed well. The mixture was incubated at -20 °C for 1–2 h. Then a small spin was given at maximum speed of 14000 rpm for 30 min and the supernatant was discarded without disturbing the pallet. The pallet was washed using about 120 µL of 70 % ethanol and given a spin at maximum speed for 5 min. The supernatant was discarded and allowed it to air dry. It was resuspended in 20 µL of autoclaved distilled water and stored at -20 °C.

Restriction fragment length polymorphism (PCR–RFLP). RFLP is an important downstream application of PCR used to detect the single nucleotide polymorphisms (SNP) in a gene. SNP is the variation of the DNA sequence which occurs if a single nucleotide A, T, G, or C differs from the members of the same species or in an individual.

The DNA sequences give rise to different cutting sites for different restriction enzymes (specific sequences for each enzyme), but due to the SNPs, it can create or destroy these regions. Accordingly, the difference in homologous DNA sequences can be detected by the presence of fragments of different lengths after digestion of the DNA samples with specific restriction endonucleases which cut at specific sequences. Amplified products are subjected to RFLP and compared with control DNA from healthy persons with normal *GH* genes. Control DNA sample are sequenced to ensure that these have the normal *GH* genes before being used in the RFLP with SmaI and NlaIII digestion.

Protocol used for the RFLP carried out with SmaI restriction enzyme. The concentrations of the purified PCR product were quantified by using specific standards with the known concentration via agarose gel electrophoresis. After the concentrations of the PCR product were determined by the agarose gel electrophoresis, the RFLP was carried out. The concentrations varied from 120 ng to 200 ng; accordingly, the amount of the PCR product differed from sample to sample and the 10 units of enzyme were used for RFLP. The reaction tubes containing the above components were kept for incubation overnight at 25 °C. After the incubation, the RFLP products (patients' samples, healthy samples and samples without the restriction enzyme) and 1000bp ladder were subjected to gel electrophoresis and the bands were visualised under the UV transilluminator.

Protocol used for the RFLP carried out with NlaIII restriction enzyme. The concentrations of the purified PCR product were quantified by using specific standards with the known concentration via agarose gel electrophoresis. After the concentrations of the PCR product were determined by the agarose gel electrophoresis, the RFLP was carried out. The concentrations varied from 120 ng to 200 ng; accordingly, the amount of the PCR product differed from sample to sample and the enzymes were used 10 units per reaction. The reaction tubes containing the above components were kept for incubation for 3–5 h at 37 °C. After the incubation, the RFLP products (patients' samples, healthy samples and samples without the restriction enzyme), and 100bp ladder were subjected to gel electrophoresis and the bands were visualised under the UV transilluminator.

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