WHOLE EXOME SEQUENCING GIVES ADDITIONAL BENEFITS COMPARED TO CANDIDATE GENE SEQUENCING IN THE MOLECULAR DIAGNOSIS OF CHILDREN WITH GROWTH HORMONE OR IGF-1 INSENSITIVITY.

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ABSTRACT

GH insensitivity (GHI) is characterised by short stature, IGF-1 deficiency and normal/elevated serum GH. IGF-1 insensitivity results in pre- and post-natal growth failure with normal/high IGF-1 levels. The prevalence of genetic defects is unknown.

Objective: To identify the underlying genetic diagnoses in a paediatric cohort with GH or IGF-1 insensitivity using candidate gene (CGS) and whole exome sequencing (WES) and assess factors associated with the discovery of a genetic defect.

Methods: We undertook a prospective study of 132 patients with short stature and suspected GH or IGF-1 insensitivity referred to our centre for genetic analysis. 107 (96 GHI, 88 probands; 11 IGF-1 insensitivity, 9 probands) underwent CGS. WES was performed in those with no defined genetic aetiology following CGS.

Results: A genetic diagnosis was discovered 38/107 (36%) patients (32% probands) by CGS. WES revealed 11 patients with genetic variants in genes known to cause short stature. A further 2 patients had hypomethylation in the H19/IGF2 region or mUPD7 consistent with Silver-Russell Syndrome (total with genetic diagnosis 51/107, 48% or 41/97, 42% probands). WES also identified homozygous putative variants in *FANCA* and *PHKB* in 2 patients. Low height SDS and consanguinity was highly predictive for identifying a genetic defect.

Conclusions: Comprehensive genetic testing confirms the genetic heterogeneity of GH/IGF-1 insensitivity and successfully identified the genetic aetiology in a significant proportion of cases. WES is rapid and may isolate genetic variants that have been missed by traditional clinically driven genetic testing. This emphasises the benefits of specialist diagnostic centres.

INTRODUCTION

Short stature is one of the most common reasons for referral to paediatric endocrinologists. Patients with defects in growth hormone (GH) action or GH insensitivity (GHI) frequently present with severe phenotypes (height SDS -≤2.5) and the aetiology often remains uncertain. Consequently, many patients are classified as having idiopathic short stature (ISS) and pose a significant diagnostic and management challenge.

The growth hormone-insulin-like growth factor-1 (GH-IGF-1) axis is essential for human growth[1]. The cardinal features of GHI are severe growth failure, normal GH secretion and IGF-1 deficiency (IGFD). Monogenic defects leading to GHI have been discovered in *GHR*[2, 3], *STAT5B*[4, 5], *IGFALS*[6], *PAPPA2*[7] and *IGF1*[8]. IGF-1 insensitivity secondary to *IGF1R* gene mutations exists as part of the continuum and leads to a similar phenotype[9]. In cases of IGF-1 resistance, the IGF-1 levels are high[10]. Depending on the genetic defect, associated clinical and dysmorphic features may be present including: mid-facial hypoplasia and frontal bossing (*GHR, STAT5B*)[4, 11], immune deficiency (*STAT5B*)[4], pubertal delay (*IGFALS, STAT5B, GHR*)[4, 11, 12], decreased bone mineral density (*PAPPA2*)[7], developmental delay, microcephaly and *in-utero* growth retardation (*IGF1, IGF1R*)[1]. 3M, Silver-Russell (SRS) and Noonan (NS) syndromes have phenotypes that can overlap with GHI[10, 13, 14]. 3M syndrome (OMIM 273750) results in pre- and post-natal growth restriction, prominent heels, facial dysmorphism and distinct radiological features[15]. The genetics are incompletely understood, but mutations in cullin 7 (*CUL7*) (70%), obscurin-like 1 (*OBSL1*) (25%) and coiled coil domain-containing 8 (*CCDC8*) (5%) genes have been identified[16, 17]. SRS is characterized by intrauterine and/or postnatal growth retardation and is caused by maternal uniparental disomy of chromosome seven (matUPD7) and hypomethylation of the imprinted H19/IGF2 domain of chromosome 11p15 in 10 and 35-65% cases, respectively[18]. Noonan syndrome results from autosomal dominant mutations in the Ras/mitogen activated protein kinase signalling pathways (*PTPN11, SOS1, SOS2, RAF1, BRAF*, *NRAS*, *KRAS, HRAS, CBL, RIT1, RASA2, MAP2K1, MAP2K2, A2ML1 LZTR1* and *SHOC2* genes) in ~70% patients[19, 20].

The identification of a pathogenic molecular defect is important for families and clinicians. A genetic diagnosis ends uncertainty, avoids unnecessary investigations and treatment, and allows appropriate genetic counselling and the identification of possible co-morbidities in syndromic short stature. A genetic diagnosis may also lead to earlier initiation of therapy and therefore a better long-term treatment response[21].

Genetic defects can be identified by traditional Sanger sequencing of the most likely candidate genes (candidate gene sequencing, CGS) or by next generation sequencing e.g. whole exome sequencing (WES). CGS is clinically driven and is reliable when the affected gene can be predicted with a high degree of certainty. Its success depends on the accurate clinical phenotyping of patients and is limited in growth disorders with overlapping, highly variable or subtle features[22]. It is also time-consuming and costly if a number of genes are analysed. In contrast, WES allows the simultaneous screening of the entire coding DNA of an individual and is therefore extremely cost effective if multiple genes are to be investigated.

As a genetic reference centre, we undertook CGS in a cohort of patients with short stature and suspected GH or IGF-1 insensitivity. This is an extension of our previous work and some of the patients have been previously reported[10]. WES was completed in patients with no diagnosis following CGS. Our data demonstrate the importance of comprehensive genetic analysis in severe short stature, particularly the utility of WES in securing a molecular diagnosis where CGS has yielded negative results.

SUBJECTS AND METHODS

Patients

Between 2008 and 2017 our centre received 132 referrals (75M) for genetic investigation. Patients were referred from: UK (n=77), Kuwait (n=20), Poland (n=10), Germany (n=6), India (n=3), Thailand (n=3), Egypt (n=2), Argentina (n=2), Turkey (n=1), Italy (n=1), Mexico (n=1), Belgium (n=1), Denmark (n=1), Sweden (n=1), Croatia (n=1), UAE (n=1) and Ireland (n=1). Patients were investigated at their home institutions and the referring physicians completed a proforma detailing the clinical and biochemical data at the time of sending the DNA sample. The referring clinicians excluded causes of secondary GHI, including undernutrition. Birth weight, height, and BMI were expressed as SDS according to the appropriate national standards. Biochemical investigations included: basal and/or peak GH and basal IGF-1 levels. IGF-1 was expressed as SDS based on the age and sex appropriate range provided by the institution. Where serum IGF-1 was undetectable (less than the lower limit of the assay) (n=17), we calculated the lowest possible detectable SDS and assigned that for the statistical analysis. In these patients, the IGF-1 SDS ranged between -2.5 and -5.3 but this is likely to underestimate the degree of IGF-1 insensitivity.

Twenty-five of 132 patients did not have the clinical and biochemical characteristics of GH or IGF-1 insensitivity and were excluded **(Figure 1A)**. Diagnoses in the excluded group included GHD (n=4), short stature associated with chromosome 10 duplication (n=1) and achondroplasia (*FGFR3* mutation) (n=1). One hundred and seven cases (97 families, 97 probands) were investigated, including 49 patients (42 probands) with consanguineous parents. Ninety-six cases (58M, median age 5.8 years, range 0.1-17.0) had features of GHI: mean height -4.5 SDS (range -9.1 to -2.0), mean IGF1 -2.9 SDS (range -8.2 to -2.0) and peak GH levels 7-1195 µg/l. A further 11 children (2M, median age 5.8 years, range 0.1-14.4) had characteristics of IGF-1 insensitivity: mean height SDS -4.1 (range -6.8 to -2.4), mean birth weight SDS -3.1 (range -5.8 to -2.0), mean IGF-1 SDS 0.7 (range -1.1 to 4.4).

Candidate gene sequencing (CGS)

Genomic DNA was isolated from peripheral blood leukocytes (Qiagen DNeasy Kit) and genetic analysis was undertaken on all patients as previously described[10]. The candidate genes sequenced

depended on the clinical and biochemical features. Most patients were screened for mutations in the growth hormone receptor gene (*GHR*); other genes were selected depending on the phenotype (**Figure 1A**). Sanger sequencing was performed by the Barts and the London Genome Centre (http://www.smd.qmul.ac.uk/gc/) or GATC Biotech (https://www.gatc-biotech.com). 2 patients underwent molecular investigations for Silver-Russell syndrome (SRS) following referral to clinical geneticists. Three patients with GHI had *STAT5B* sequencing.

Whole Exome Sequencing (WES)

WES was completed in 54 patients (53 probands and 11 unaffected relatives) who had no genetic cause for their short stature identified by CGS (**Figure 1A**). The remaining 15 patients did not consent for WES.

Twenty-three patients and 3 relatives were processed using the Agilent SureSelect all exon V4 capture and paired-end (2 x 100) sequencing on an Illumina HiSeq 2000 at Otogenetics (Norcross, GA). 31 patients and 8 relatives were sequenced using SureSelect Human All Exon v5 (51Mb) capture and paired-end (2 x 100) sequencing on an Illumina 2500 Standard run (minimum coverage 50x) at Oxford Gene Technology (OGT, Oxford, UK). >90% of target bases were covered 10X. For comparison, WES data from 43 in-house controls generated on the same platforms were analyzed by the same pipeline described below.

Variant analysis

The raw data from Otogenetics or OGT were analysed using DNA Nexus (DNAnexus Inc., Mountain View, CA, USA) by aligning to the H. sapiens GRCh37–b37 (1000 genomes Phase 1) reference genome with BWA-MEM FastQ Readmapper VCF files, generated by Vendor Human Exome GATK-Lite Variant Caller (Unified Genotyper). The resulting VCF files were uploaded to Ingenuity Variant Analysis (Qiagen, Germany). The following filter settings were applied: call quality was set to ≥20 and read depth ≥10 and only data outside 0.1% of most exonically variable 100 base windows in healthy

public genomes and outside 0.1% most exonically variable genes in healthy public genomes (1000 genomes, ExAC) were included. Common variants were filtered out by excluding those with an allele frequency of ≥0.1% in the 1000 genomes, ExAC and the NHLBI exomes. Missense variations that were classified as loss of function by Ingenuity were included i.e. the amino acid change was predicted to affect function and those that were predicted benign were excluded. Variants that passed these filters and were predicted damaging by either SIFT or PolyPhen were explored further (**Figure 1B**):

Analysis 1: Variants were sought in 22 genes known to cause features of GHI or IGF-1 insensitivity (*GHR*, *IGFALS, STAT5B, IGF1, PAPPA2*, *IGF1R, OBSL1, CCDC8, CUL7, PTPN11, SOS1, SOS2, RAF1, BRAF*, *NRAS*, *KRAS, HRAS, CBL, RIT1, NF1, LZTR1* and *SHOC2)*. Genetic variants were confirmed by Sanger sequencing (SS), primer sequences available on request. Forty-five family members underwent SS to assess the segregation of the variant within family structures. If no putative causal variants were found we progressed to analysis 2.

A*nalysis 2*: Variants were sought in 153 biological candidate genes associated with: syndromic growth disorders, skeletal dysplasias, growth plate biology, cell proliferation, DNA repair or growth retardation in mice (**Suppl. Table 1**). An autosomal recessive model was adopted i.e. homozygous, hemizyous (for X-linkage) or potentially compound heterozygous variants as there were no affected parents. Variants were only included if they were present in patients and absent in controls. Since the cohort is genetically and phenotypically heterogeneous, we hypothesized that the same causal variant was unlikely to be seen in multiple patients (barring related individuals). Therefore, any variants that were present in ≥3 patients were discarded. If no putative causal variants were identified by these criteria we progressed to analysis 3.

A*nalysis 3*: Variants were sought in novel candidate genes by an unbiased approach, seeking homozygous or putative compound heterozygous variants. Novel candidate genes were included if

predicted deleterious variants were identified in ≥2 patients and were absent in controls. Although this strategy may miss private mutations, it provides corroborative evidence that the gene is implicated in the phenotype. As above, identical variants that were present in ≥3 patients were discarded. Candidate genes satisfying these bioinformatic criteria were investigated *in silico* (see novel variants).

Rare variant burden testing

Rare variant burden testing was applied to the pre-filtered variants from analysis 3 to identify genes enriched for rare variants in patients but not controls. The following script was employed using freeware R (https://cran.r-project.org/):

```
Table<-read.Table(file="genes.txt",head=FALSE) #imports file genes
apply(Table,1, function(Table) fisher.test(matrix(Table,nr=2))$p.value)
```

Novel variants

Novel variants were investigated *in silico* by SIFT (score ranges from 0 (predicted deleterious) to 1 (predicted benign)), PolyPhen-2 (score ranges from 0 (predicted benign) to 1 (predicted deleterious)), Variant effect predictor (VEP), Mutation Taster and Human Splicing Finder (HSF version 3.0) to predict the functional outcome. VEP defines the likely deleterious effect of the variant as low, moderate, or high. Mutation Taster predicts whether the variant is predicted disease causing or benign. HSF predicts whether a variant makes exon skipping more likely than the reference allele. PubMed, OMIM and String determined Gene function and for pathway analysis.

Statistical analysis

The differences in height SDS, IGF-1 SDS and peak GH between those with and without an identified genetic defect were analysed using an unpaired t-test. Univariate logistic regression analysis identified predictor variables (SPSS version 22; IBM Corp. Armonk, NY).

Ethics

Informed written consent for genetic research was obtained from patients and/or their parents.

RESULTS

Diagnosis by Candidate Gene Sequencing (CGS)

CGS identified likely causative variants in 35 GHI patients (28 probands) and 3 IGF-1 insensitivity patients, all probands (total 38/107; 36% or 31/97, 32% probands) **(Table 1)**. These included variants in *GHR (n=27* patients*)*, *IGFALS (n=3* patients*)*, *OBSL1 (n=6* patients*)*, *CUL7 (n=1* patients*)* and *IGF1R (n=1* patient*)* **(Figures 1A and 2).** 30 of 38 (79%) children diagnosed by CGS had consanguineous parents. *STAT5B* sequencing was normal in the 3 patients tested.

GHR: Fifteen *GHR* variants (5 novel and 10 previously described) were identified in 27 patients (patients 1-27, **Table 1**) with mean serum IGF-1 -3.5 SDS (range -8.5 to -2.3), mean basal and peak GH concentrations 40.7 µg/l (range 1.8 to 398, n=21) and 123.2 µg/l (range 15.7–1195, n=20). All had homozygous variants in *GHR* with the exception of 2 patients (21 & 26) who had compound heterozygous variants, inheriting one defective allele from each parent. All children except patient 1 had a "classical" Laron syndrome phenotype. The most commonly identified *GHR* defect was the homozygous *6ψ* mutation (c.618+792A>G, p.Met206_Met207ins36) in patients 1-8 of UK Pakistani or Indian origin. These included two unrelated pairs of siblings (patients 2 & 3, 5 & 6) and four other non-familial cases (patients 1, 4, 7, 8). Patient 1 had a *GHR* intronic pseudoexon *(6ψ)* mutation with characteristic features of GHI (height SDS -4.0, IGF1 SDS -2.6, peak GH levels 119 µg/l) but no dysmorphic features. Four of the novel *GHR* variants were homozygous; c.198C>A (p.Cys66*), c.700C>T (p.Gln234*), c.599A>G (p.Asn200Ser) c.344A>C (p.Asn115Thr) all predicted deleterious by at least one functional outcome prediction method. Patient 21 had 2 previously described variants in compound heterozygosity (c.266+83G>T (p.?) and (c.723C>T, p.Gly241_Glu261del). The final novel

variant, c.922G>A (p.Gly308Arg) predicted deleterious by SIFT, was found in compound heterozygosity with a known *GHR* variant in patient 26. Other known *GHR* mutations identified were: c.740T>C (p.Leu247Pro), c.594A>G (p.Glu198*), c.785-6 T>A (p.Asp264Glyfx*), c.247C>T (p.Gln83*), c.703C>T (p.Arg235*), c.439+1 G>A (p.Arg89Serfs*47), c.723C>T (p.Gly241_Glu261del) and c.168C>A (p.Cys56*).

IGFALS: 3 GHI patients (28-30) had homozygous *IGFALS* variants (mean serum IGF1 SDS -2.7 (-3.6 to -1.9) and mean peak GH concentration 20.5 µg/L (16.0 to 28.9 µg/L). One variant c.1291delT, pTrp431Glyfs*11 has been previously described [10]. Interestingly, the previously described p.Leu134Gln variant identified in 2 patients (28 & 29) was associated with SGA but no dysmorphic features[10].

3M syndrome genes: We identified 2 previously described homozygous *OBSL1* mutations c.1463C>T (p.Arg489*) (patients 31 and 32) and c.1359insA, (p.Glu454Argfs*) (patients 33-36) and 1 homozygous *CUL7* c.2710C>T (p.Arg904*) mutation (patient 37) [23, 24]. All patients had consanguineous parents. All patients had severe short stature (mean height SDS -5.8) with normal GH (mean peak GH 21.8). Most had severe IGF-1 deficiency but 2 (patients 35 & 37) had IGF-1 levels of -0.2 and -0.25, respectively. Additional but variable clinical features of the 3M syndrome were present in all 7 patients **(Table 1)**.

IGF1R: A heterozygous missense variant was identified in one patient with an IGF-1 insensitivity phenotype (birth weight -2.7 SDS, height SDS -3.1, IGF-1 SDS 2.0, basal GH 17.5 µg/l) (patient 38). This heterozygous variant, c.112G>A, (p.Asp38Asn) has previously been described **(Table 1)**[10].

Silver-Russell syndrome

Hypomethylation in the imprinting control region 11p15 and mUPD7 was demonstrated in patients 39 and 40, respectively. Both had features of GHI as previously described (frontal bossing, mid-facial hypoplasia, height SDS -3.7 and -4.3, and IGF-1 SDS -2.8 and -3.4) **(Figures 1A and Suppl. Table 2)**[10].

Diagnosis by Whole Exome Sequencing (WES) (Figure 1B)

164,113 variants in 18,476 genes were called in 54 patient exomes (53 probands). Following the application of the filters described above for true rare predicted deleterious changes, this reduced to 11,912 variants in 9,849 genes.

Analysis 1 **(Table 2 and Figure 2)***:* 11/54 patients (20%) (10 probands, 19%) were found to have variants in genes known to cause GHI (homozygous *GHR* (n=5), compound heterozygous *IGFALS* (n=1), homozygous *CCDC8* (n=1), homozygous *CUL7* (n=1), heterozygous *PTPN11* (n=2) and heterozygous *SOS*1 (n=1)).

GHR: Patients 47-51 **(Table 2)** with *GHR* variants had classical Laron phenotypes (mean height SDS -5.1, mean IGF-1 SDS -4.7 and mean peak GH 46.8 µg/l). Patients 47-50 had a novel homozygous GHR variant c.70+4A>C (p.?) (exon skipping predicted by HSF) and were from consanguineous families of Kuwaiti origin, therefore a founder effect is likely. Patient 51 had a previously described *GHR* c.703C>T, p.Arg235* (R217X) mutation, and had a clinical picture of classical Laron syndrome with height SDS -5.9, IGF-1 of -5.3, and peak GH >35.

IGFALS: Patient 46 **(Table 2)** with novel compound heterozygous *IGFALS* variants c.1576G>A (p.Asp526Asn) and c.632G>A, (p.Trp211*), both predicted deleterious (SIFT score 0), had a typical phenotype (height SDS -5.0, IGF1 SDS -2.5 and peak GH 13 µg/L).

Noonan syndrome (NS) genes: Patients 41 and 42 had previously described heterozygous *PTPN11* c.417G>C (p.Glu139Asp) and c.853T>C (p.Phe285Leu) mutations [25]. Both had short stature (height

SDS -2.1 and -3.1), IGF-1 deficiency (-2.3 and-2.4), dysmorphic features and were SGA (birth weight SDS -2.1 and -3.0). The phenotype of the parents of patient 41 is unknown and we do not have parental DNA. The mother of patient 42 has the same variant and a clinical phenotype of NS. Patient 43 had isolated short stature and a novel heterozygous c.3418T>A (p.Leu1140Ile) *SOS1* variant predicted disease causing by Mutation taster **(Table 2)**. This patient's father also has a similar phenotype of isolated short stature (-2.4 SDS) but parental DNA was not available to confirm the segregation.

3M syndrome genes: Patients 44 and 45 had previously observed defects in *CCDC8* (c.612dupG, p.Lys205Glufs*59) and *CUL7* (c.2988G>A, p.Trp996*), respectively and had a classical GHI phenotype **(Table 2).**

Analysis 2: 43 remaining patients (all probands; 38 with GHI and 5 with IGF-1 insensitivity) were screened for variants in 153 biological candidate growth genes associated with: syndromic growth disorders, skeletal dysplasias, growth plate biology, cell proliferation, DNA repair or growth retardation in mice (**Suppl. Table 1**). A homozygous variant was identified in one patient in *FANCA* (c.2000C>G, p.P667R; mother heterozygous, paternal DNA not available) predicted damaging by SIFT and probably damaging by PolyPhen. A homozygous variant was identified in one patient in *PHKB* (c.56-1G>A; adopted child therefore parental DNA not available), which is associated with glycogen storage disease type IX (GSD IX). This alters one of the canonical splice site bases and is likely to cause exon skipping and an aberrant protein. 2 variants were identified in *MDC1* (c.3774_3775delGCinsAT, p.P1259S and c.3528_3529delGCinsAT, p.P1177S; both predicted tolerated/benign by PolyPhen and SIFT) in one patient and 2 variants in another patient in *EVC2* (c.673G>T, p.A225S ; and c.664T>A, p.F222I; possibly and probably deleterious by PolyPhen, respectively) which were inherited together in cis from one parent who has normal stature. The *FANCA* and *PHKB* variants are potential candidates to explain the phenotype in 2 patients. In

contrast, due to the *in silico* predictions and mode of inheritance respectively, the *MDC1* and *EVC2* variants were presumed non-pathogenic.

Analysis 3: In light of the dearth of variants identified by analysis 2, WES data from all 43 undiagnosed patients were investigated using an unbiased approach. This strategy produced a shortlist of 109 variants in 77 candidate genes. Variants in all 77 genes were seen in GHI patients but only 4 genes had variants in patients with IGF-1 insensitivity (*in **Supplementary Table 3**), none of which were specific to IGF-1 insensitivity. PubMed and OMIM did not reveal obvious growth associations of the 77 candidate genes and pathway analysis did not reveal any enriched functional pathways. On rare variant burden testing, none of the 77 candidate genes were found to be significantly enriched for deleterious variants in cases vs controls. Therefore, the significance of these variants is uncertain.

Associations between phenotypic features and genetic defects

Although there was significant overlap, patients with identified genetic defects were significantly shorter compared to those with no genetic diagnosis (mean height SDS -5.2 vs -3.7; p<0.0001) (**Figure 3**). Height SDS was significantly lower in patients with *GHR* or 3M gene mutations compared to individuals with no genetic diagnosis (both p<0.0001) (**Table 3).** IGF-1 SDS values were significantly lower in patients with any genetic defect and in those with *GHR* mutations compared to individuals with no genetic diagnosis (p=0.0128 and <0.0001, respectively). GH levels were obtained from a number of different referral centres and likely measured by more than one assay. However, taking this limitation into account, peak GH levels were significantly higher in patients with *GHR* mutations compared to those with no genetic diagnosis (p=0.0177) (**Table 3**). Patients with *GHR 6Ψ* mutations had less severe phenotypes when compared to patients with other homozygous *GHR* defects (Mean height SDS -4.07 vs -6.2 respectively, p=0.0006) as previously described[26]. Consanguinity was predictive for identifying a molecular defect but age and sex were not (**Suppl. Table 4**).

DISCUSSION

In approximately 80% of patients with short stature the aetiology remains elusive despite detailed clinical, biochemical and radiological assessment[27]. This includes patients with extreme or syndromic short stature. Growth hormone (GHI) and IGF-1 insensitivity encompass a spectrum of clinical and biochemical abnormalities associated with normal GH secretion[1]. The degree of short stature is variable in this group of disorders but in many cases the growth failure is severe.

The majority of referrals to our genetic sequencing service were male as previously described[10]. Our cohort was heterogeneous but all patients had a phenotype consistent with GH or IGF-1 insensitivity i.e. short stature (height SDS ≤-2.0), GH sufficiency (peak GH ≥7.0 µg/L) and low or normal/elevated IGF-1 levels, respectively.

Multiple mutations have been discovered in the GH-IGF-1 axis in association with GH and IGF-1 insensitivity including mutations in the *GH1, GHR, STAT5B, IGFALS, PAPPA2, IGF1 and IGF1R* genes[1, 7]. We recently noted that, as well as the classically recognised GH-IGF-1 axis gene defects, other short stature disorders may have features of GHI such as 3M, Noonan and Silver-Russell (SRS) syndromes[10]. Consequently, we now routinely screen GHI patients born small for gestational age (SGA) for mutations in the 3M syndrome genes (*OBSL1*, *CUL7* and *CCDC8*) as well as *IGF1*. Genetic testing for SRS was not carried out on other undiagnosed SGA subjects in this cohort; therefore, it is possible that other cases of SRS might have been missed. A proportion of short patients may carry disease-causing copy number variation (CNVs) or gene deletions / microdeletions[28] and analyses to detect this are currently underway in our undiagnosed patients. As such, deletions of candidate genes may have been missed by our analysis. Although detecting CNVs from WES is challenging, the use of algorithms may facilitate this process [29].

Candidate gene sequencing (CGS) using Sanger sequencing is based on the selection of appropriate gene(s) for analysis depending on the patient's clinical phenotype and hormonal profile. This approach is reliant on accurate clinical information available at the time of referral and is usually restricted to a small number of genes due to the time and cost implications. In contrast, next generation sequencing techniques such as targeted gene panels can be employed to analyse all genes known to cause a genetically heterogeneous disorder in one test. Alternatively, whole exome sequencing (WES), allows the simultaneous analysis of all genes. Although gene panels can be powerful diagnostic tools, the advantage of WES is that data can also be mined for deleterious variants in novel genes not previously linked with a disease. Today, WES can be undertaken with a relatively low cost, however the interpretation of results can be difficult in inexperienced hands and the coverage of genes can be variable.

The traditional (CGS) approach alone confirmed a diagnosis in 35% of our cohort (31% probands); the majority of cases (92%) were diagnosed following sequencing of 1 or 2 genes. This technique is therefore relatively reliable if the phenotype is accurately documented and is typical for the disorder e.g. extreme short stature and IGF-1 deficiency (IGFD) with classical Laron syndrome features[10, 30]. Interestingly, we isolated a further 8 genetic variants in 11 patients in GHI genes by WES. These were not initially detected by CGS either because the variant was outside of the region amplified by Sanger sequencing in the case of the novel homozygous *GHR* gene mutation identified in 4 Kuwaiti patients or the phenotype was atypical (*IGFALS, PTPN11, SOS1, CCDC8, CUL7*). In the final Kuwaiti patient, the homozygous *GHR* mutation had been missed as a result of human error. Clinical phenotyping can be challenging for even experienced clinicians and many conditions have a wide phenotypic spectrum. In retrospect, the referring clinicians identified clinical features associated with Noonan and 3M syndromes in the 2 patients with previously reported *PTPN11* mutations and the patients with *CUL7* and *OBSL1* mutations, respectively. The patient with a novel heterozygous

401 *SOS1* gene variant was born SGA, had short stature and IGFD but no classical features of NS. The
402 other patient with novel compound heterozygous *IGFALS* gene variants is shorter (-5.0 SDS) than
403 most/all previously reported patients with IGFALS defects[1]. This emphasizes not only the importance
404 of accurate clinical phenotyping prior to referral for genetic testing but also the difficulties in
405 diagnosing many short stature syndromes. Noonan in particular, should be carefully considered
406 when assessing a patient with features of GHI[31].
407
408 Eleven novel genetic variants were identified in *GHR*, *IGFALS* and *SOS1* genes. As functional studies
409 were not undertaken on the novel variants, it remains a possibility that they are not responsible for
410 the clinical phenotype. However, familial segregation and *in silico* prediction programs have been
411 utilized to substantiate them. Except for cases 42 (compound heterozygous *IGFALS*) and 45
412 (heterozygous *SOS1*) the phenotypes are also typical for the identified genetic defects [1]. Therefore,
413 we are confident that these genetic variants explain the clinical presentation. According to ExAC, the
414 *SOS1* gene is intolerant of loss of function variants (expected number of loss of function variants
415 57.5; observed loss of function variants 3, pLI = 1.0) this increases the likelihood of this variant being
416 pathogenic. The *IGFALS* variants are both predicted to be highly deleterious and the patient had
417 reduced birth weight (SDS -3.4). Together these factors may contribute to the development of a
418 more extreme phenotype. No dysmorphic features or other potential genetic variants in candidate
419 genes were identified in this patient that could explain the more severe phenotype. However, we
420 cannot rule out oligogenicity with a novel gene defect. Prenatal growth retardation in particular has
421 been previously recognized to contribute to the heterogeneity of IGFALS defects[32].
422
423 We also identified a novel homozygous, predicted deleterious *FANCA* mutation in a patient with
424 normal birth weight, short stature (-3.0 SDS) and IGFD (-2.0). Fanconi anaemia (FA), is an autosomal
425 recessive trait, associated with skeletal and cardiac defects, pre- and post-natal growth retardation
426 and malformation of the kidneys, although consistent with this case, 25% patients have no reported
427 physical abnormalities[33]. The mean age at presentation is typical (usually ~7 yrs) and short stature is

recognised presenting feature in children[33]. Chromosome breakage test with mitomycin C (MMC) did not show any spontaneous chromosome fragility. Unfortunately, a lack of chromosomal fragility does not exclude FA and further investigations are currently underway. GSD IX is caused by *PHKB* mutations resulting in phosphorylase kinase deficiency. The novel, predicted damaging homozygous mutation was identified in a child with severe short stature (-4.5 SDS) and IGFD (-4.1 SDS). *PHKB* has an autosomal recessive mode of inheritance and the symptoms, severity and prognosis are highly variable, even among individuals with the same mutation. Characteristic features include, hepatomegaly, hypotonia, fasting hypoglycaemia and growth / pubertal delay. Although growth delays can be pronounced in affected children, catch-up growth is common and normal adult height is usually attained[34]. This patient is under investigation by the local metabolic team.

The identification of FA and GSD in children is crucial to initiate close monitoring for serious long-term complications (haematological malignancies / hepatic and cardiac, respectively) and studies are underway to validate these diagnoses. Ideally, functional studies should be undertaken to definitively attribute the *FANCA* and *PHKB* mutations to the phenotypes. Due to the *in silico* predictions and mode of inheritance respectively, the *MDC1* and *EVC2* variants were presumed non-pathogenic and have not been further investigated. The molecular diagnosis of all but 2 patients in the cohort could have potentially been secured using a next generation sequencing panel encompassing the genes included in Analysis 2. However, the advantage of WES is that it may serendipitously reveal a serious paediatric disorder, such as FA or GSD, which may have longer-term medical implications. Additionally, a gene panel would need to be continuously updated as further genetic causes of short stature are discovered. Furthermore, the cost of WES is significantly cheaper than undertaking CGS of the 22 genes known to cause GH and IGF-1 insensitivity (Analysis 1) i.e. approximately £600 vs £1750.

The identification novel genetic causes of short stature is essential to advance our understanding and management of growth disorders. To address this we used an unbiased approach (Analysis 3) to

uncover variants in genes, which might represent novel aetiologies for short stature. No strong candidate gene(s) emerged from this analysis but we hereby report the results for reference. The failure to identify other genes may be a result of wider genetic heterogeneity i.e. numerous undiscovered genes which make a major contribution to growth exist which cannot be identified in our relatively small cohort. Oligogenic inheritance of genes known to cause short stature may also explain some short stature phenotypes, although Analysis 2 does not support this. It is also possible that a combination of both these factors may be important. As we were unable to perform trio analysis on all patients, we may have missed some *de novo* variants acting in a dominant fashion. Additionally, CNV or unexplored e.g. intronic or regulatory regions of the known genes not covered by WES, such as the *GHR* pseudoexon mutation, may contribute [26, 28]. In the coming years, whole genome sequencing will uncover more such examples.

Deciding which short patients to refer for genetic testing can be problematic. Knowledge of the clinical features associated with different gene mutations is key to deciding which gene to prioritize. Our data suggests that accurate assessment of height, IGF-1 and GH may improve the diagnostic yield. Additionally, a genetic defect is more likely to be identified in consanguineous offspring. The current study suggests that CGS is reliable when the clinical features and the biochemical profile strongly suggest a particular candidate gene e.g. a *GHR* mutation. However, if a genetic diagnosis is not secured following sequencing of two candidate genes, then the CGS strategy is unlikely to reveal a genetic diagnosis and it is also more cost effective to proceed to WES.

We present the results of comprehensive genetic testing in a cohort of patients with GH and IGF-1 insensitivity. A number of novel defects were identified in several genes associated with GH and IGF insensitivity. Our data expand the phenotypes associated with several genetic defects and also the spectrum of overlapping diagnoses associated with GHI. Next generation sequencing is an important adjuvant to CGS in the diagnosis of genetic short stature and emphasises the benefit of specialist diagnostic centres.

URLs

Ingenuity: http://www.ingenuity.com/

freeware R: https://cran.r-project.org/

HSF: http://www.umd.be/HSF3/

Mutation taster: www.mutationtaster.org

VEP: http://grch37.ensembl.org/Homo_sapiens/Tools/VEP

SIFT: http://sift.jcvi.org

PolyPhen: http://genetics.bwh.harvard.edu/pph2/

Pubmed: https://www.ncbi.nlm.nih.gov/pubmed/

OMIM: http://www.omim.org/

String: http://string-db.org

ExAC: http://exac.broadinstitute.org

HGVS: http://varnomen.hgvs.org/

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Author contribution statement: LAM and LS performed bioinformatics analyses. SC, DGR, KMD and HLS contributed to patient recruitment, data collection and analysis. SC performed the phenotypic and statistical analyses. HLS wrote the manuscript with input from SC, LAM, LS and DGR.

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FIGURE TITLES AND LEGENDS

Figure 1A. Summary of candidate gene (CGS) and whole exome sequencing (WES) in the GH and IGF-1 insensitivity patients.

3M syndrome genes, *CUL7*, *CCDC8* and *OBSL1;* BW, birth weight.*The candidate genes sequenced depended on the clinical and biochemical features. The majority of patients were screened for mutations in the growth hormone receptor gene (*GHR*) +/- *IGFALS*. Other genes were selected depending on the phenotype e.g. *STAT5B* if there was evidence of immune deficiency / eczema / atopy and *IGF1* and 3M genes if birth weight SDS was ≤ 2.0 SDS.

Figure 1B. Whole exome sequencing (WES) data analyses: number of patients assessed and variants identified.

Figure 2. Genetic Diagnoses in the GH and IGF-1 insensitivity patients.

Figure 3. Height SDS in patients with a genetic diagnosis and those with no genetic diagnosis.

Diagnosed patients n=50, undiagnosed n=55; ★ p = 0.0001

Table 1. Clinical, biochemical and genetic features of patients diagnosed by candidate gene sequencing (CGS) (total 37 patients, 39 variants)

Pt no.	Age (yr)	Sex	Consang-uinity /ethnicity	Birth weight SDS	Height SDS	BMI SDS	Target Height SDS	GH basal (µg/L)	GH max (µg/L)	IGF-1 (ng/ml)	IGF-1 SDS	Clinical features	Genetic variants	MAF ExAC	Predicted outcome (novel variants)	Reference	No. genes analysed by CGS
													***GHR* gene**				
1	8.0	M	+/Pakistani	-0.5	-4.0	0.7	-0.9	13.2	119.0	18.2	-2.6	No	Hom c.618+792A>G, p.Met206_Met207ins36	0	-	Metherell *et al* 2001[26]	1 (*GHR*)
2^{S}	4.2	F	+/Pakistani	0.1	-4.2	-1.0	0.7	16.3	33.3	<22.4	-2.5[a]	Classical	Hom c.618+792A>G, p.Met206_Met207ins36	0	-	Metherell *et al* 2001[26]	1 (*GHR*)
3^{S}	7.5	M	+/Pakistani	-2.9	-4.5	-1.2	-1.3	4.0	>33	1.4	-2.8	Classical	Hom c.618+792A>G, p.Met206_Met207ins36	0	-	Metherell *et al* 2001[26]	1 (*GHR*)
4	7.7	M	+/Indian	-1.7	-3.1	-2.4	-1.9	3.2	30.3	11.2	-2.6	Classical	Hom c.618+792A>G, p.Met206_Met207ins36	0	-	Metherell *et al* 2001[26]	1 (*GHR*)
5^{B}	14.7	M	+/Pakistani	0.7	-3.0	-0.7	-1.0	11.3	39.6	9.1	-3.1	Classical	Hom c.618+792A>G, p.Met206_Met207ins36	0	-	Metherell *et al* 2001[26]	1 (*GHR*)
6^{B}	2.3	M	+/Pakistani	NK	-4.7	-0.5	-1.0	50.8	46.0	<22.4	-3.1[a]	Classical	Hom c.618+792A>G, p.Met206_Met207ins36	0	-	Metherell *et al* 2001[26]	1 (*GHR*)
7	2.4	F	+/Pakistani	-1.8	-5.0	-0.4	N/D	3.4	26.7	134.3	-2.3	Classical	Hom c.618+792A>G, p.Met206_Met207ins36	0	-	Metherell *et al* 2001[26]	1 (*GHR*)
8	6.8	F	+/ Pakistani	-0.3	-4.1	-0.2	-0.9	56.1	30.3	30.3	-4.0	Classical	Hom c.618+792A>G, p.Met206_Met2	0	-	Metherell *et al* 2001[26]	1 (*GHR*)

													07ins36				
9[b]	2.4	M	-/ Argentinian	0.2	-7.7	-0.8	-1.9	NK	57.0	UD	-2.5[a]	Classical	**Hom c.198C>A, p.Cys66***	0	High impact (VEP)	Unpublished	1 (*GHR*)
10[b]	14.3	M	-/ Argentinian	0.5	-8.4	0.0	-1.9	NK	88.0	9.0	NK	Classical	**Hom c.198C>A, p.Cys66***	0	High impact (VEP)	Unpublished	1 (*GHR*)
11	8.4	M	+/Turkish	-6.0	-8.7	1.8	-1.9	NK	79.0	5.1	NK	Classical	**Hom c.700C>T, p.Gln234***	0	High impact (VEP)	Unpublished	1 (*GHR*)
12	5.8	M	+/NK	-0.7	-7.7	-0.7	-1.4	NK	33443 44A32. 5	UD	-2.5[a]	Classical	Hom c.740T>C, p.Leu247Pro (p.L229P)	0	Deleterious (SIFT score 0)	Khan *et al*, 2009[35]	1 (*GHR*)
13	NK	M	NK/Mexican	NK	NK	NK	NK	NK	NK	NK	NK	Classical	Hom c.594A>G, p.Glu198* (E180X)	0	-	Berg *et al,* 1992[36]	1 (*GHR*)
14	4.2	M	+/ Bangladeshi	1.6	-6.9	-5.6	-1.8	398.0	1195.0	<25	-2.5[a]	Classical	Hom c.785-6 T>A, p.Asp264Glyfx* 5 (p.D244GfsX5)	0	-	David *et al,* 2010[37]	1 (*GHR*)
15	1.8	F	+/ Bangladeshi	-0.2	-5.7	-2.2	-2.3	36.0	ND	<25	-2.5[a]	Classical	Hom c.247C>T, p.Gln83* (Q65X)	0	-	Sobrier *et al,* 1997[38]	1 (*GHR*)
16	1.5	F	+/ Bangladeshi	-1.2	-6.1	0.7	-2.0	60.0	ND	<25	-2.5[a]	Classical	Hom c.703C>T, p.Arg235* (R217X)	8.2x E^{-6} ɣ	-	Amselem *et al,* 1993[39]	1 (*GHR*)
17	1.1	M	+/Kuwaiti	2.6	-5.0	0.8	-0.3	>47	>47	<30	-2.5[a]	Classical	Hom c.703C>T, p.Arg235* (R217X)	8.2x E^{-6} ɣ	-	Amselem *et al,* 1993[39]	1 (*GHR*)
18	5.7	M	+/ Kuwaiti	-0.6	-5.3	1.7	-1.3	14.4	35.0	32.9	-2.5[a]	Classical	Hom c.703C>T, p.Arg235* (R217X)	8.2x E^{-6} ɣ	-	Amselem *et al,* 1993[39]	1 (*GHR*)
19[c]	9.4	M	+/Egyptian	2.1	-6.4	-5.5	-1.5	16.0	33.3	9.5	-8.5	Classical	Hom c.439+1 G>A, p.Arg89Serfs*47	0	-	Storr *et al,* 2015[10]	1 (*GHR*)
20[c]	6.2	M	+/Egyptian	2.1	-5.5	-2.8	-1.5	1.8	15.7	8.1	-8.2	Classical	Hom c.439+1 G>A, p.Arg89Serfs*47	0	-	Storr *et al,* 2015[10]	1 (*GHR*)
21	10.5	M	-/Caucasian	1.5	-2.9	1.8	1.0	42.2	ND	42.0	-3.0	Classical	c.266+83G>T, p.?	0	-	Feigerlova *et al* [40]	1 (*GHR*)
													c.723C>T,	0	-	Baumbach	

												p.Gly241_Glu261del (p.G223_E243del)			*et al,* 1997[41]	
22[d]	15.3	F	-/Thai	-2.3	-9.1	-1.3	-2.15	ND	41	67.5	-2.5	Classical	Hom. c.723C>T, p.Gly241_Glu261del (p.G223_E243del)	0	-	Baumbach *et al,* 1997[41]	1 (*GHR*)
23[d]	5.8	F	-/Thai	-1.8	-6.9	-1.0	-2.15	15.9	108.44	<1	-2.8[a]	Classical	Hom. c.723C>T, p.Gly241_Glu261del (p.G223_E243del)	0	-	Baumbach *et al,* 1997[41]	1 (*GHR*)
24[e]	4.0	M	+/Indian	-1.3	-7.9	-1.3	-2.62	12.4	ND	<25	-3.1[a]	Classical	**Hom c.599A>G, p.Asn200Ser**	0	Deleterious (SIFT)	Unpublished	1 (*GHR*)
25[e]	1.3	M	+/Indian	-2.6	-8.9	-2.0	-2.62	24.5	ND	ND	ND	Classical	**Hom c.599A>G, p.Asn200Ser**	0	Deleterious (SIFT)	Unpublished	1 (*GHR*)
26	1.6	F	-/Caucasian	-1.4	-4.3	-1.4	NK	25.5	ND	<25	-2.5[a]	Classical	c.168C>A, p.Cys56* (p.C38X)	8.2x E^{-6} [γ]	-	Amselem *et al,* 1991[42]	1 (*GHR*)
													c.922G>A, p.Gly308Arg	0	Deleterious (SIFT score 0)	Unpublished	
27	3.1	F	+/Arabic-Syrian	-1.1	-4.5	-1.2	-1.02	3.3	75.0	8.0	-7.3	Classical	**Hom c.344A>C, p.Asn115Thr**	0	Deleterious (SIFT)	Unpublished	1 (*GHR*)
													***IGFALS* gene**				
28[s]	8.0	F	+/Indian	-3.0	-3.9	-2.5	-2.32	2.2	28.9	<16.0	-2.5[a]	No	Hom c.401T>A p.Leu134Gln (p.L134Q)		-	David *et al,* 2010[43]	2 (*GHR, IGFALS*)
29[s]	6.1	M	+/Indian	-2.1	-2.0	NK	-4.1	1.1	16.7	25.9	-1.9	No	Hom c.401T>A p.Leu134Gln (p.L134Q)		-	David *et al,* 2010[43]	1 (*IGFALS*)
30	13.5	F	+/Moroccan	-1.3	-3.3	-1.75	-1.63	9.7	16.01	66.0	-3.6	No	Hom c.1291delT, pTrp431Glyfs*11 (p.W431Gfs11)	0	-	Storr *et al,* 2015[10]	2 (*GHR, IGFALS*)

												3M syndrome genes				
31	4.6	M	+/Bedouin	-3.2	-7.4	-0.7	-0.5	6.0	32	6.4	-2.5	Classical	*OBSL1* Hom c.1463C>T, p.Arg489* (p.R489X)	0	-	Hanson *et al,* 2009[23]	2 (*IGF1R, OBSL1*)
32	1.0	M	+/Kuwaiti	-1.6	-6.4	-2.3	-1.3	2.1	18.2	30.5	-2.5	Classical Hypermobility, prominent heels	*OBSL1* Hom c.1463C>T p.Arg489* (p.R489X)	0	-	Hanson *et al,* 2009[23]	4 (*GHR, IGFALS, IGF1, OBSL1*)
33	3.0	F	+/Kuwaiti	-5.2	-5.7	-4.7	NK	9.1	15	<3.0	-2.7[a]	Classical Hypermobility, prominent heels	*OBSL1* Hom c.1359insA, p.Glu454Argfs*11 (p.E454RfsX11)	0	-	Hanson *et al,* 2009[23]	2 (*IGF1, OBSL1*)
34[g]	1.1	F	+/Kuwaiti	-3.8	-4.9	-0.4	-0.34	4.2	37.2	ND	ND	Classical. Hypermobility	*OBSL1* Hom c.1359insA, p.Glu454Argfs*11(p.E454RfsX11)	0	-	Hanson *et al,* 2009[23]	2 (*IGF1, OBSL1*)
35[g]	0.1	F	+/Kuwaiti	-2.6	-5.1	0.7	-0.34	5.4	10.8	105.0	-0.2	Classical Prominent heels	*OBSL1* Hom c.1359insA, p.Glu454Argfs*11(p.E454RfsX11)	0	-	Hanson *et al,* 2009[23]	2 (*IGF1R, OBSL1*)
36	0.06	F	+/Kuwaiti	-1.5	-4.5	0.5	-1.2	41.0	33	<27	-2.6	Classical hypermobility, prominent heels short fingers, trident hands, short rib cage,	*OBSL1* Hom c.1359insA, p.E454Rfs*11 (p.E454RfsX11)	0	-	Hanson *et al,* 2009[23]	4 (*GHR, IGFALS, IGF1, OBSL1*)

												bilateral DDH					
37	10.8	F	+/ Bangladeshi	-2.9	-6.8	1.0	-2.39	NK	6.5	159.0	-0.3	Disproportion large head, short limbs, lumbar lordosis	*CUL7* Hom c.2710C>T, p.Arg904*	0	-	Huber *et al*, 2010[44]	3 (*IGF1R, OBSL1, CUL7*)
													***IGF1R* gene**				
38	6.6	F	-/Caucasian	-2.7	-3.1	-1.3	-0.46	17.5	9.6	367.0	2.0	Triangular face, long fingers	Het c.112G>A, p.Asp38Asn (p.D38N)	0	-	Storr *et al,* 2015[10]	1 (*IGF1R*)

Novel genetic variants are in bold font. Height SDS is at presentation. ND, not done; NK, not known; UD, undetectable; + parents consanguineous; -, parents not consanguineous; classical, classical GHI phenotype (frontal bossing, mid-facial hypoplasia); No, no dysmorphic features; DDH, developmental dysplasia of the hip; a, IGF-1 level less than the lower limit of the assay (SDS -2.5); Hom, homozygous; Het, heterozygous; VEP, variant effect predictor; S/s, siblings; B/b/c/e, brothers; d/g, sisters. 3M syndrome genes, *CUL7*, *CCDC8* and *OBSL1*. c. coding DNA sequence where nucleotide 1 is the A of the ATG-translation initiation codon, for *GHR* the transcript includes exon 3 NCBI Reference Sequences NM_000163; for *IGFALS* NM_004970; for *CUL7* NM_014780*;* for *OBSL1* NM_015311*;* for *IGF1R* NM_000875; **, p.Met188_Met189ins36 mutation aka pseudoexon activation (6Ψ); ins, insertion; fs, frameshift; *, termination site; as, acceptor site; ds, donor site; X, stop codon; del, deletion; $^{\beta}$ predicted result if exon is skipped. c. coding DNA sequence where nucleotide 1 is the A of the ATG-translation initiation codon of *OBSL1* gene, NCBI reference NM_015311.2; MAF, minor allele frequency - variants are defined as rare if the MAF is <0.001 (0.1%) as recorded on the ExAC (Exome Aggregation Consortium) database; $^{\gamma}$, no homozygotes in ExAC database. Variation predicted by HSF, HSF predicts exon skipping to be more likely than in reference allele; SIFT score 0 is deleterious, 1 is benign. References refer to the genetic variants. No. genes sequenced by CGS, number of genes (and which genes) sequenced by candidate gene sequencing (CGS) before a diagnosis was made. Variant nomenclature is according to the HGVS guidelines. Italicised patient numbers indicate those patients previously reported in Storr *et al,* 2015[10].

Table 2. Clinical, biochemical and genetic features of patients diagnosed by whole exome sequencing (WES) (total 11 patients, 12 variants)

Pt no.	Age (yr)	Sex	Consang-uinity /ethnicity	Birth weight SDS	Height SDS	BMI SDS	Target Height SDS	GH basal (µg/L)	GH max (µg/L)	IGF-1 (ng/ml)	IGF-1 SDS	Clinical features	Genetic variants	MAF ExAC	Predicted outcome (novel variants)	Reference	No. genes analysed by CGS
													Noonan syndrome genes				
41	6.9	M	+/Kuwaiti	0.3	-2.1	-2.7	-1.5	1.1	>32	35.4	-2.3	Low set ears, undescended left testis	*PTPN11* Het c.417G>C p.Glu139Asp (p.E139D)	0	-	Tartaglia *et al*, 2002[25]	1 (*GHR*)
42	8.9	F	-/Polish	-2.1	-3.2	-1.6	0.6	21.7	10.5	47	-2.4	Low set ears, hypertelorism, mild ptosis, low posterior hairline	*PTPN11* Het c.853T>C, p.Phe285Leu (p.F285L)	0	-	Tartaglia *et al*, 2002[25]	1 (*GHR*)
43	13.1	M	-/Mexican-Russian	-3.0	-3.8	-1.5	-1.5	0.4	26.6	7.4	-2.63	No	***SOS1*** **Het c.3418T>A p.Leu1140Ile**	3.5x E^{-5} ¥	Disease causing (Mutation Taster)	Unpublished	2 (*GHR, IGFALS*)
													3M syndrome genes				
44	1.9	F	+/Pakistani	-3.5	-5.7	-1.6	N/D	3.0	5.0	7.5	-1.8	Classical	*CCDC8* Hom c.612dupG, p.Lys205Glufs*59 (p.Lys205GlufsX59)	1.8x E^{-5} ¥	-	Hanson *et al,* 2009[23]	1 (*GHR*)
45	0.3	F	+/Kuwaiti	-5.8	-5.5	-0.6	NK	22.5	26.7	116	-1.1	Classical bilateral DDH	*CUL7* Hom c.2988G>A, p.Trp996* (pW996X)	0	-	Al-Dosari *et al,* 2012[45]	1 (*GHR*)

												***IGFALS* gene**				
46	15.4	F	+/ Pakistani	-3.4	-5.0	1.0	-3.4	0.1	13	35	-2.5	Classical	**c.1576G>A p.Asp526Asn**	1.7x E^{-4} ɣ	Deleterious (SIFT score 0)	Unpublished	1 (*GHR*)
													c.632G>A, p.Trp211*	0	Deleterious (SIFT score 0)	Unpublished	
													***GHR* gene**				
47[h]	3.5	F	+/ Kuwaiti	-0.82	-3.9	-1.2	-1.8	9.5	85	<30	-5.3	Classical	**Hom c.70+4A>C, p.?**	0	Variation predicted by HSF	Unpublished	1 (*GHR*)
48[h]	2.2	F	+/ Kuwaiti	-0.34	-2.5	-0.4	1.8	22	ND	<30	-5.3	Classical	**Hom c.70+4A>C, p.?**	0	Variation predicted by HSF	Unpublished	1 (*GHR*)
49	2.0	F	+/ Kuwaiti	-2.1	-6.7	-2.3	0.3	35	>35	<30	-5.3	Classical	**Hom c.70+4A>C, p.?**	0	Variation predicted by HSF	Unpublished	1 (*GHR*)
50	1.2	F	+/ Kuwaiti	+0.12	-6.7	-1.3	-1.4	11.3	>32	<10	-2.1	Classical	**Hom c.70+4A>C, p.?**	0	Variation predicted by HSF	Unpublished	1 (*GHR*)
51	1.4	M	+/ Kuwaiti	+1.99	-5.9	-0.2	-0.1	>35	>35	<30	-5.3	Classical	Hom. c.703C>T, p.Arg235* (R217X)	8.2x E^{-6} ɣ	-	Amselem *et al,* 1993[39]	1 (*GHR*)

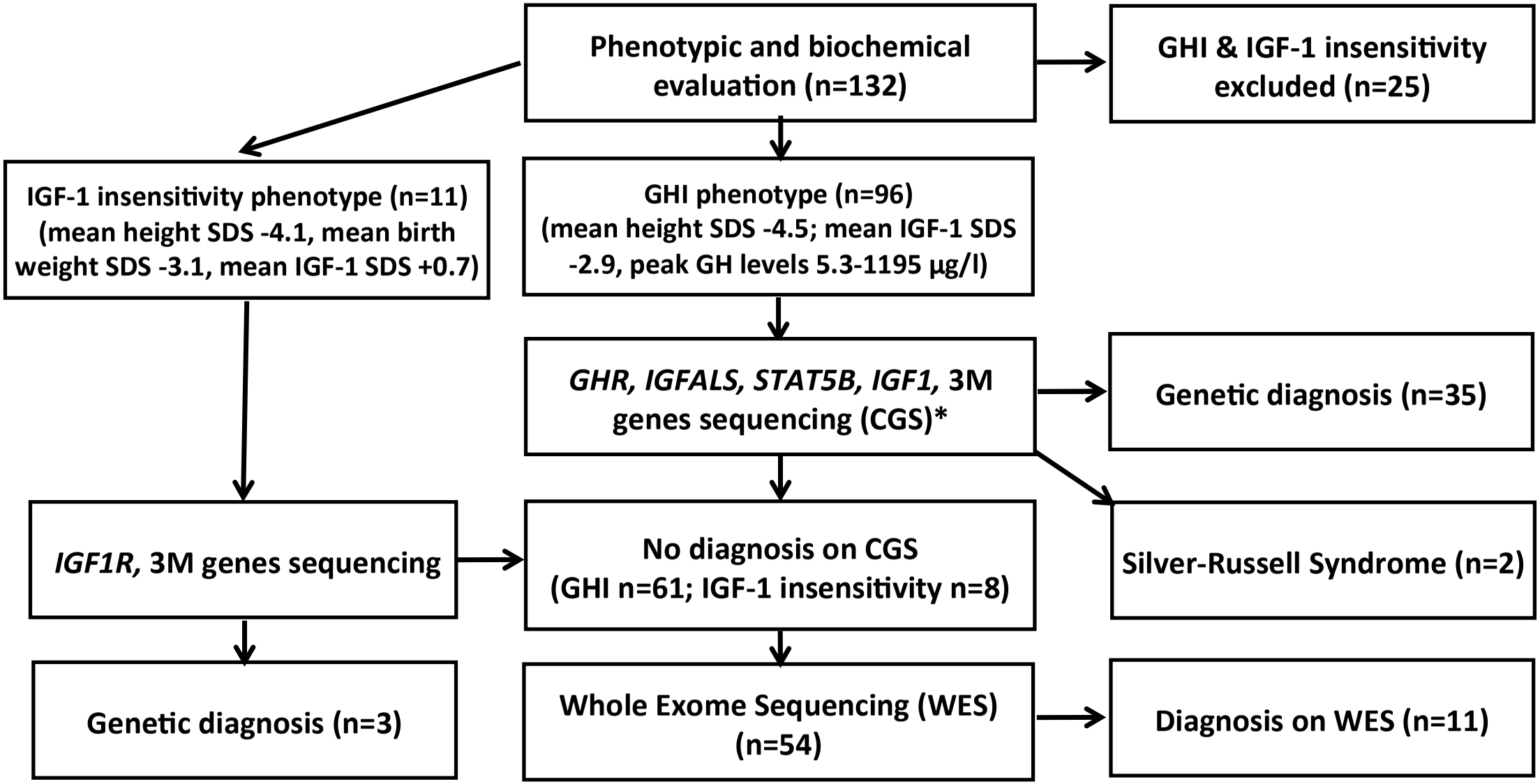
Novel genetic variants are in bold font. Patients that underwent WES had no genetic diagnosis obtained following candidate gene sequencing. Height SDS is at presentation. ND, not done; + parents consanguineous; -, parents not consanguineous; classical, classical GHI phenotype (frontal bossing, mid-facial hypoplasia); No, no dysmorphic features; DDH, developmental dysplasia of the hip; Hom, homozygous; Het, heterozygous; VEP, variant effect predictor; h, sisters. 3M syndrome genes, *CUL7*, *CCDC8* and *OBSL1*. Noonan syndrome genes*, PTPN11*, *SOS1*. c. coding DNA sequence where nucleotide 1 is the A of the ATG-translation initiation codon, for *GHR* the transcript includes exon 3 NCBI Reference Sequences NM_000163; for *IGFALS* NM_004970; for *CUL7* NM_014780*;* for *CCDC8* NM_032040*;* for *SOS1* NM_005633; *PTPN11* NM_001330437; ins, insertion; fs, frameshift; *, termination site; as, acceptor site; ds, donor site; X, stop codon; del, deletion; [β] predicted result if exon is skipped. c. coding DNA sequence where

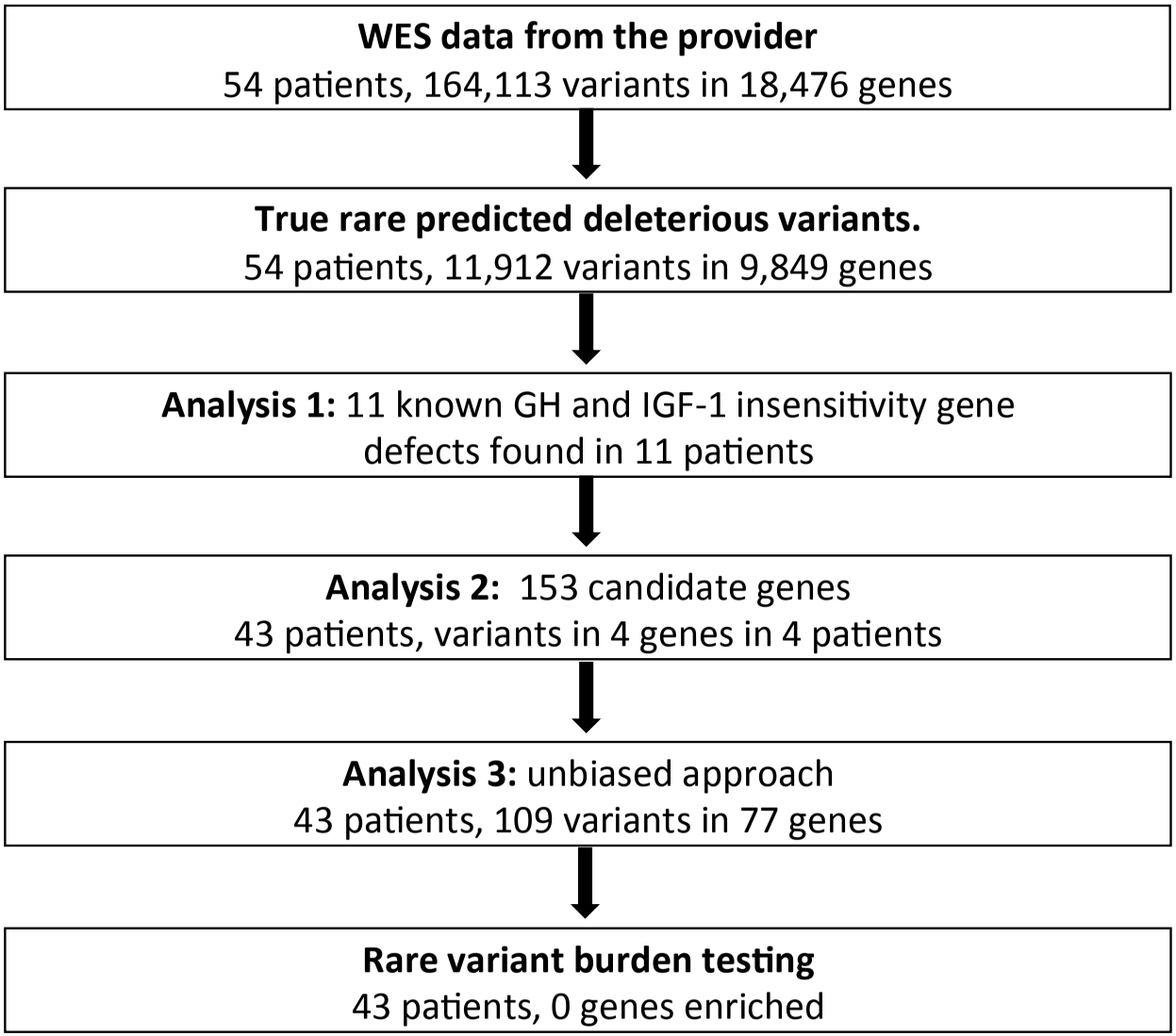
nucleotide 1 is the A of the ATG-translation initiation codon of *OBSL1* gene, NCBI reference NM_015311.2; MAF, minor allele frequency - variants are defined as rare if the MAF is <0.001 (0.1%) as recorded on the ExAC (Exome Aggregation Consortium) database; [v], no homozygotes in ExAC database. Variation predicted by HSF, HSF predicts exon skipping to be more likely than in reference allele; SIFT score 0 is deleterious, 1 is benign. References refer to the genetic variants. No. genes sequenced by CGS, number of genes (and which genes) sequenced by candidate gene sequencing (CGS) before proceeding to whole exome sequencing (WES). Variant nomenclature is according to the HGVS guidelines.

Table 3. Comparison of mean height SDS, IGF-1 SDS and peak GH levels between individuals with genetic defects and those with no molecular diagnosis

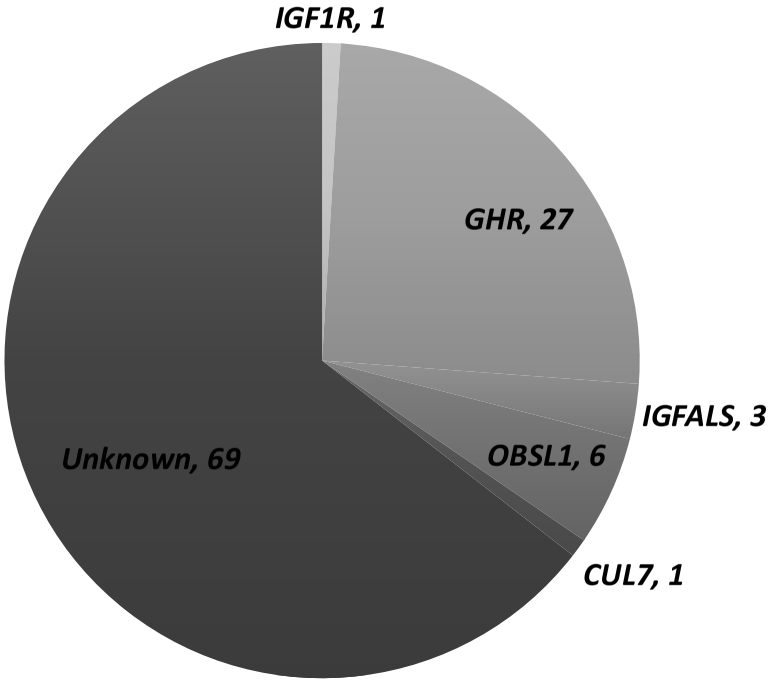
	No genetic diagnosis (Group 1)	*GHR* and *GHR* 6Ψ mutations (Group 2)	3M gene mutations (Group 3)	Any genetic diagnosis (Group 4)	Group 1 vs Group 2 P value (95% CI)	Group 1 vs Group 3 P value (95% CI)	Group 1 vs Group 4 P value (95% CI)
Mean height SDS★	-3.7 ± 1.2 (n=50)	-5.7 ± 1.9 (n=31)	-5.7 ± 0.9 (n=9)	-5.2 ± 1.8 (n=51)	**<0.0001** (1.3 to 2.6)	**<0.0001** (1.2 to 2.8)	**<0.0001** (0.89 to 2.1)
Mean IGF-1 SDS★	-2.1 ± 1.5 (n=48)	-3.7 ± 1.9 (n=26)	-1.6 ± 1.0 (n=8)	-3.0± 2.0 (n=42)	**<0.0001** (0.87 to 2.45)	0.3682 (-1.6 to 0.6)	**0.0128** (0.2 to 1.63)
Mean peak GH ★	21.6± 17.1 (n=51)	93.7 ± 212.3 (n=31)	21.4 ± 13.4 (n=9)	68.1 ± 173.3 (n=48)	**0.0177** (12.9 to 131.3)	0.9676 (-12.3 to 11.8)	0.0594 (-1.88 to 94.9)

★Means ± S.D.; CI, confidence intervals; 3M gene mutations, mutations identified in *CUL7*, *CCDC8* and *OBSL1.*


Phenotypic and biochemical evaluation (n=132)
GHI & IGF-1 insensitivity excluded (n=25)
IGF-1 insensitivity phenotype (n=11) (mean height SDS -4.1, mean birth weight SDS -3.1, mean IGF-1 SDS +0.7)
GHI phenotype (n=96) (mean height SDS -4.5; mean IGF-1 SDS -2.9, peak GH levels 5.3-1195 µg/l)
GHR, IGFALS, STAT5B, IGF1, 3M genes sequencing (CGS)*
Genetic diagnosis (n=35)
IGF1R, 3M genes sequencing
No diagnosis on CGS (GHI n=61; IGF-1 insensitivity n=8)
Silver-Russell Syndrome (n=2)
Genetic diagnosis (n=3)
Whole Exome Sequencing (WES) (n=54)
Diagnosis on WES (n=11)

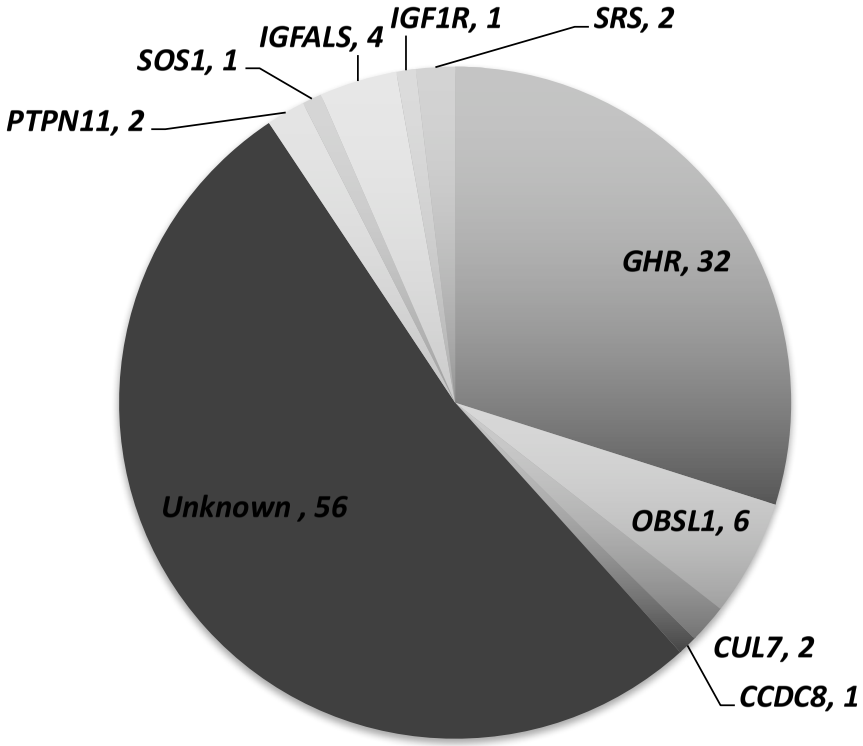

WES data from the provider
54 patients, 164,113 variants in 18,476 genes
True rare predicted deleterious variants.
54 patients, 11,912 variants in 9,849 genes
Analysis 1: 11 known GH and IGF-1 insensitivity gene defects found in 11 patients
Analysis 2: 153 candidate genes
43 patients, variants in 4 genes in 4 patients
Analysis 3: unbiased approach
43 patients, 109 variants in 77 genes
Rare variant burden testing
43 patients, 0 genes enriched

CGS

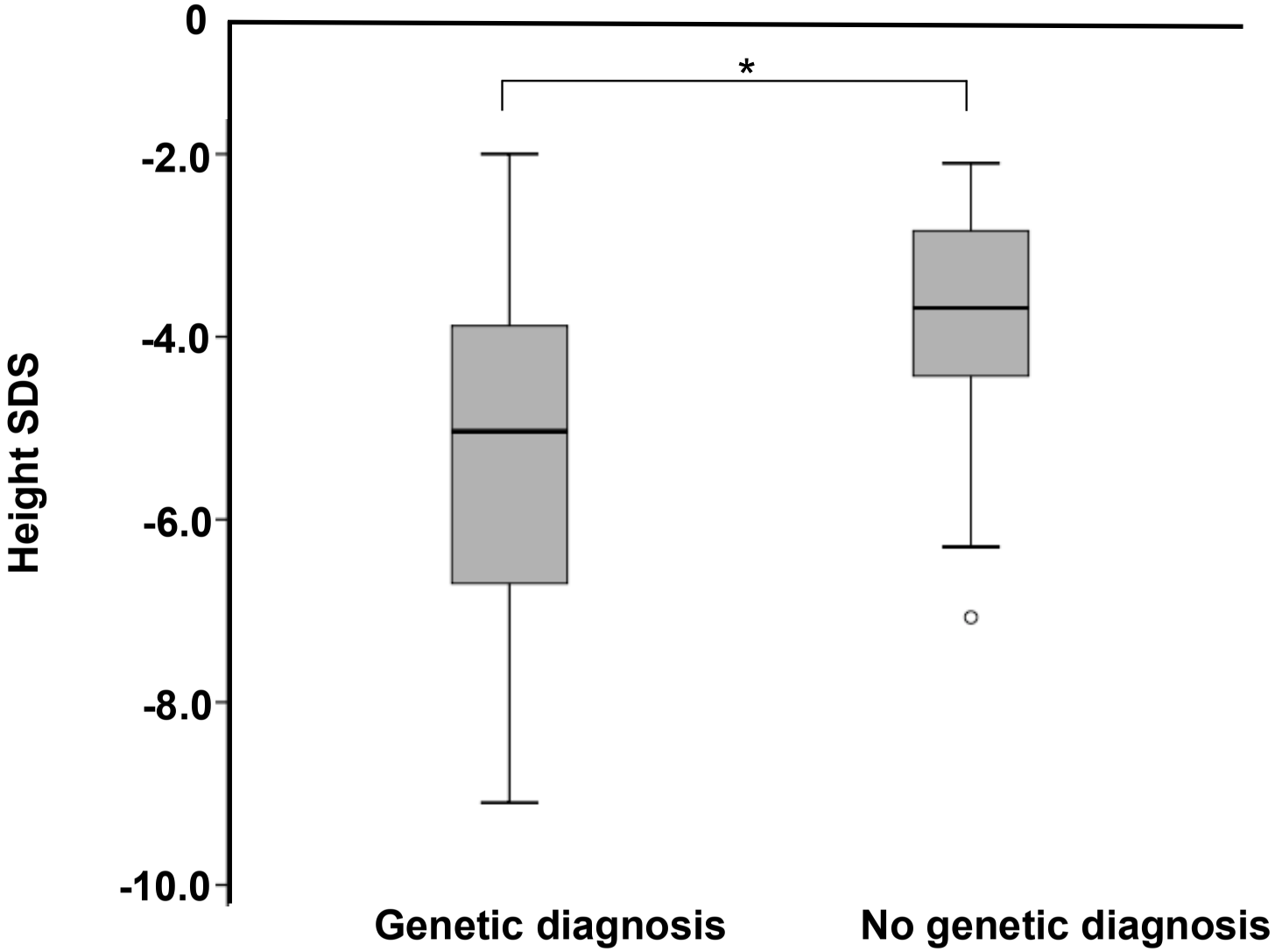


(38/107) 36%

All modalities



(51/107) 48%


0
-2.0
-4.0
-6.0
-8.0
-10.0
Height SDS
*
Genetic diagnosis
No genetic diagnosis

Supplementary Table 1. List of biological candidate genes and their functional roles for genetic variant Analysis 2 (n=153)

Gene	Functional roles / associated disease
ADAM12	ADAM metallopeptidase domain 12; Involved in skeletal muscle regeneration, specifically at the onset of cell fusion.
ACAN	AGGRECAN 1; Aggrecan is a major component of cartilage extracellular matrix. Defects are associated with short stature
AGL	amylo-alpha-1, 6-glucosidase, 4-alpha-glucanotransferase. Glycogen storage disease and associated growth retardation
AKT1	AKT1 is one of 3 closely related serine/threonine- protein kinases (AKT1, AKT2 and AKT3) called the AKT kinase, and which regulate many processes including metabolism, proliferation, cell survival, growth and angiogenesis.
AKT2	v-akt murine thymoma viral oncogene homolog 2; AKT2 is one of 3 closely related serine/threonine- protein kinases (AKT1, AKT2 and AKT3) called the AKT kinase, and which regulate many processes including metabolism, proliferation, cell survival, growth and angiogenesis.
AKT3	AKT3 is one of 3 closely related serine/threonine- protein kinases (AKT1, AKT2 and AKT3) called the AKT kinase, and which regulate many processes including metabolism, proliferation, cell survival, growth and angiogenesis.
ANKRD11	ankyrin repeat domain 11; KBG syndrome and associated short stature
ATR	Acting as a DNA damage sensor. Recognizes the substrate consensus sequence [ST]-Q. Phosphorylates BRCA1, CHEK1, MCM2, RAD17, RPA2, SMC1 and p53/TP53, which collectively inhibit DNA replication and mitosis and promote DNA repair, recombination and apoptosis.
ATRIP	ATR interacting protein ATRIP is phosphorylated by ATR, regulates ATR expression, and is an essential component of the DNA damage checkpoint pathway
B3GAT3	Beta-1,3-glucuronyltransferase 3; Multiple joint dislocations, short stature, craniofacial dysmorphism, with or without congenital heart defects.
B4GALT7	Xylosylprotein 4-beta-galactosyltransferase, polypeptide 7; defects cause Ehlers-Danlos syndrome with short stature and limb anomalies.
BLM	Bloom syndrome, RecQ helicase-like; Participates in DNA replication and repair. Exhibits a magnesium-dependent ATP-dependent DNA-helicase activity that unwinds single- and double-stranded DNA in a 3'-5' direction. Involved in 5'-end resection of DNA during double-strand break (DSB) repair.
BMP6	bone morphogenetic protein 6; Induces cartilage and bone formation
BOD1L1	Biorientation of chromosomes in cell division 1-like 1; Recognition and repair of damaged replication forks is essential for maintenance of genome stability. BOD1L is a component of the fork protection pathway that responds to replication stress.
CDC6	Cell division cycle 6 homolog (S. cerevisiae); Involved in the initiation of DNA replication. Also participates in checkpoint controls that ensure DNA replication is completed before mitosis is initiated.

CDK1	Cyclin-dependent kinase 1; Plays a key role in the control of the eukaryotic cell cycle by modulating the centrosome cycle as well as mitotic onset; promotes G2-M transition, and regulates G1 progress and G1-S transition via association with multiple interphase cyclins.
CDKN1B	Cyclin-dependent kinase inhibitor. The HER2-HER3 dimer induces cell growth by activating a kinase cascade that includes phosphorylation of CDKN1B, resulting in CDKN1B ubiquitination and proteasomal degradation.
CDKN1C	Cyclin-dependent kinase inhibitor 1C (p57, Kip2); IMAGe syndrome - severe IUGR and marked postnatal growth failure.
CDT1	Chromatin licensing and DNA replication factor 1; Cooperates with CDC6 to promote the loading of the mini- chromosome maintenance complex onto chromatin to form the pre- replication complex necessary to initiate DNA replication. Meier-Gorlin syndrome 4 associated with short stature.
CENPJ	Centromere protein J; Plays an important role in cell division and centrosome function by participating in centriole duplication. Inhibits microtubule nucleation from the centrosome. Seckel syndrome 4 - intrauterine and postnatal growth retardation.
CEP152	Centrosomal protein 152kDa; Regulator of genomic integrity and cellular response to DNA damage acting through ATR-mediated checkpoint signaling. Necessary for centrosome duplication. It functions as a molecular scaffold facilitating the interaction of PLK4 and CENPJ, two molecules involved in centriole formation.
CEP63	Centrosomal protein 63kDa; Required for normal spindle assembly. Maintains centrosome numbers through centrosomal recruitment of CEP152. Also recruits CDK1 to centrosomes. Plays a role in DNA damage response. (By similarity).
CHD7	Chromodomain helicase DNA binding protein 7; Probable transcription regulator. CHARGE syndrome, retardation of growth.
CHEK1	Checkpoint kinase 1; Serine/threonine-protein kinase which is required for activation of DNA repair in response to the presence of DNA damage or unreplicated DNA.
COL2A1	Collagen, type II, alpha 1. Type II collagen, also called cartilage collagen, is the major collagen synthesized by chondrocytes.
COL27A1	Collagen, type xxvii, alpha-1; Steel syndrome: dislocated hips and radial heads, carpal coalition, scoliosis, and short stature.
CREBBP	CREB binding protein; Rubinstein-Taybi syndrome - growth retardation.
DGCR8	DiGeorge syndrome critical region gene 8.
DLK1	Delta-like 1 homolog (Drosophila); Phenotype is characterized by prenatal and postnatal growth retardation.
DPH1	*DPH1*, S. cerevisiae, homolog of; Developmental delay with short stature, dysmorphic features, and sparse hair.
EGR1	Early growth response 1; Transcriptional regulator. Recognizes and binds to the DNA sequence 5'-CGCCCCCGC-3'(EGR-site). Activates the transcription of target genes whose products are required for mitogenesis and differentiation.

EVC2	Ellis van Creveld syndrome 2; Positive regulator of the hedgehog signalling pathway (By similarity). Plays a critical role in bone formation and skeletal development.
FANCA	Fanconi anemia, complementation group A; Clinical manifestations of Fanconi anemia include pre- and postnatal growth retardation; malformations of the kidneys, heart, and skeleton.
FANCC	Fanconi anemia, complementation group C; DNA repair protein that may operate in a postreplication repair or a cell cycle checkpoint function. May be implicated in interstrand DNA cross-link repair and in the maintenance of normal chromosome stability.
FANCD2	Fanconi anemia, complementation group D2; Required for maintenance of chromosomal stability. Promotes accurate and efficient pairing of homologs during meiosis. Involved in the repair of DNA double-strand breaks, both by homologous recombination and single-strand annealing. May participate in S phase and G2 phase checkpoint activation upon DNA damage. Plays a role in preventing breakage and loss of missegregating chromatin at the end of cell division, particularly after replication stress.
FANCG	Fanconi anemia, complementation group G; DNA repair protein that may operate in a postreplication repair or a cell cycle checkpoint function. May be implicated in interstrand DNA cross-link repair and in the maintenance of normal chromosome stability.
FBN1	Fibrillin 1; Geleophysic Dysplasia 2 and Acromicric Dysplasia, both associated with short stature
FBXW8	F-box and WD repeat domain containing 8; Substrate-recognition component of a SCF-like E3 ubiquitin-protein ligase complex, which mediates the ubiquitination and subsequent proteasomal degradation of target proteins. In complex with CUL7, mediates ubiquitination and consequent degradation of GORASP1, acting as a component of the ubiquitin ligase pathway.
FGD1	FYVE, RhoGEF and PH domain containing 1; Aarskog-Scott syndrome associated with short stature.
FGF8	Fibroblast growth factor 8 (androgen-induced); Plays an important role in the regulation of embryonic development, cell proliferation, cell differentiation and cell migration.
FGFR3	Fibroblast growth factor receptor 3; Tyrosine-protein kinase that acts as cell-surface receptor for fibroblast growth factors and plays an essential role in the regulation of cell proliferation, differentiation and apoptosis. Plays an essential role in the regulation of chondrocyte differentiation, proliferation and apoptosis, and is required for normal skeleton development. Regulates both osteogenesis and postnatal bone mineralization by osteoblasts.
FHL2	Four and a half LIM domains 2; May function as a molecular transmitter linking various signalling pathways to transcriptional regulation. Negatively regulates the transcriptional repressor E4F1 and may function in cell growth.
FOXRED1	FAD-dependent oxidoreductase domain-containing protein 1; Leigh syndrome, mitochondrial complex deficiency 1. Associated with a number of clinical features including intrauterine growth retardation, marked growth and developmental delay in humans.
G6PC	Glucose-6-phosphatase, catalytic subunit; Glycogen storage disease (short stature is the feature in 90% patients) Hydrolyzes glucose-6-phosphate to glucose in the endoplasmic reticulum. Forms with the glucose-6-phosphate

	transporter (SLC37A4/G6PT) the complex responsible for glucose production through glycogenolysis and gluconeogenesis. Hence, it is the key enzyme in homeostatic regulation of blood glucose levels.
GAA	Glucosidase, alpha; acid; (short stature is the feature in 90% patients). Essential for the degradation of glygogen to glucose in lysosomes.
GBA	Glucosidase, beta, acid; Gaucher disease, includes subnormal growth velocity. Lysosomal storage disorder due to deficient activity of beta-glucocerebrosidase.
GBE1	Glucan (1,4-alpha-), branching enzyme 1; Required for sufficient glycogen accumulation. The alpha 1-6 branches of glycogen play an important role in increasing the solubility of the molecule and, consequently, in reducing the osmotic pressure within cells. Glycogen storage disease with stunted growth.
GH1	Growth Hormone
GHRHR	Growth hormone releasing hormone receptor; Receptor for GRF, coupled to G proteins which activate adenylyl cyclase. Stimulates somatotroph cell growth, growth hormone gene transcription and growth hormone secretion.
GHSR	Growth hormone secretagogue receptor; Receptor for ghrelin, coupled to G-alpha-11 proteins. Stimulates growth hormone secretion. Binds also other growth hormone releasing peptides (GHRP) (e.g. Met-enkephalin and GHRP-6) as well as non-peptide, low molecular weight secretagogues (e.g. L-692,429, MK-0677, adenosine).
GLI2	GLI family zinc finger 2; acts as a transcriptional activator. May play a role during embryogenesis. Mutations cause Culler-Jones Syndrome which includes short stature secondary to hypopituitarism with growth hormone deficiency
GLI3	GLI-Kruppel family member 3; Pallister-Hall syndrome is a pleiotropic disorder comprising hypothalamic hamartoma, pituitary dysfunction, central polydactyly, and visceral malformations.
NGEF	Guanosine nucleotide exchange factor; Part of the RAS signalling pathway: Adaptor-GNEF complex translocates to the membrane where GNEF activates Ras.
GRB10	Growth factor receptor-bound protein 10.
GRB2	Growth factor receptor-bound protein 2; Adapter protein that provides a critical link between cell surface growth factor receptors and the Ras signaling pathway.
GSC	Goosecoid homeobox; Short stature, auditory canal atresia, mandibular hypoplasia, skeletal abnormalities
GYS1	Glycogen synthase 1 (muscle); Transfers the glycosyl residue from UDP-Glc to the non- reducing end of alpha-1,4-glucan. Glycogen storage disease.
GYS2	Glycogen synthase 2 (liver); Transfers the glycosyl residue from UDP-Glc to the non- reducing end of alpha-1,4-glucan. Glycogen storage disease.
HESX1	HESX homeobox 1; Required for the normal development of the forebrain, eyes and other anterior structures such as the olfactory placodes and pituitary gland. Growth hormone deficiency with pituitary anomalies.
HMGA2	High mobility group AT-hook 2; Functions as a transcriptional regulator. Functions in cell cycle regulation through CCNA2. Plays an important role in chromosome condensation during the meiotic G2/M transition of

	spermatocyte HMGA2 functioning is required for human growth and development.
HRAS	V-HA-RAS Harvey rat sarcoma viral oncogene homolog; Growth factor signalling pathway. Defects cause Costello syndrome is a rare multiple congenital anomaly syndrome associated in all cases with a characteristic coarse facies, short stature, distinctive hand posture and appearance, severe feeding difficulty, and failure to thrive.
IGF2	Insulin-like growth factor 2.
IGF2R	Insulin-like growth factor 2 receptor; This receptor binds IGF2, defects in which likely cause severe growth restriction with distinctive facies.
IGFBP1	Insulin-like growth factor binding protein 1; IGF-binding proteins prolong the half-life of the IGFs and have been shown to either inhibit or stimulate the growth promoting effects of the IGFs on cell culture. They alter the interaction of IGFs with their cell surface receptors. Promotes cell migration.
IGFBP2	Insulin-like growth factor binding protein 2, 36kDa; Inhibits IGF-mediated growth and developmental rates. IGF-binding proteins prolong the half-life of the IGFs and have been shown to either inhibit or stimulate the growth promoting effects of the IGFs on cell culture. They alter the interaction of IGFs with their cell surface receptors.
IGFBP3	Insulin-like growth factor binding protein 3; IGF-binding proteins prolong the half-life of the IGFs and have been shown to either inhibit or stimulate the growth promoting effects of the IGFs on cell culture. They alter the interaction of IGFs with their cell surface receptors. Also exhibits IGF-independent antiproliferative and apoptotic effects mediated by its receptor TMEM219/IGFBP-3R.
IGFBP4	Insulin-like growth factor binding protein 4; IGF-binding proteins prolong the half-life of the IGFs and have been shown to either inhibit or stimulate the growth promoting effects of the IGFs on cell culture. They alter the interaction of IGFs with their cell surface receptors.
IGFBP5	Insulin-like growth factor binding protein 5; IGF-binding proteins prolong the half-life of the IGFs and have been shown to either inhibit or stimulate the growth promoting effects of the IGFs on cell culture. They alter the interaction of IGFs with their cell surface receptors.
IGFBP6	Insulin-like growth factor binding protein 6; IGF-binding proteins prolong the half-life of the IGFs and have been shown to either inhibit or stimulate the growth promoting effects of the IGFs on cell culture. They alter the interaction of IGFs with their cell surface receptors.
IRS1	Insulin receptor substrate 1; May mediate the control of various cellular processes by insulin. IRS1 acts as an interface between signalling proteins with Src homology-2 domains and the receptors for insulin, IGF2, growth hormone, several interleukins, and other cytokines. Downregulation of IRS1 inhibited cell growth in HEK293 and breast cancer cells by suppressing cycle cycle progression from G0/G1 to S phase CHICO, a Drosophila homolog of the vertebrate IRS gene family, plays an essential role in the control of cell size and growth.
IRS2	Insulin receptor substrate 2; Insulin receptor substrates (IRS proteins) mediate the pleiotropic effects of insulin and insulin-like growth factor-1, including regulation of glucose homeostasis and cell growth and survival.The experiments showed that Irs1 and Irs2 are critical for embryonic and

	postnatal growth, with Irs1 having the predominant role.
JAK2	Janus kinase 2; Non-receptor tyrosine kinase involved in various processes such as cell growth, development, differentiation or histone modifications. Mediates essential signaling events in both innate and adaptive immunity. In the cytoplasm, plays a pivotal role in signal transduction via its association with type I receptors such as growth hormone (GHR).
KAL1	Kallmann syndrome 1 sequence; Kallmann syndrome has been reported to be associated with short stature.
KDM5C	Lysine-specific demethylase 5C; X-linked mental retardation and short stature, Claes-Jensen type
KDM6A	Lysine (K)-specific demethylase 6A; Kabuki syndrome. All of the patients with KDM6A mutations had short stature and postnatal growth retardation.
LHX3	LIM homeobox 3;A mouse recessive mutation called 'stubby' (stb) maps to the same area on chromosome 2 as the Lhx3 gene. Homozygous stb mice exhibit disproportionate dwarfing, manifested in shorter than normal head, body, and legs. Patients with mutations in LHX3 were reported to have combined pituitary deficiency and skeletal abnormalities.
LHX4	LIM homeobox 4; 4 affected members of a French family with LHX4 mutation had short stature, pituitary and cerebellar defects, and abnormalities of the sella turcica of the central skull base.
LIG1	Ligase I, DNA, ATP-dependent; DNA ligase that seals nicks in double-stranded DNA during DNA replication, DNA recombination and DNA repair.
LIG4	Ligase IV, DNA, ATP-dependent; Efficiently joins single-strand breaks in a double- stranded polydeoxynucleotide in an ATP-dependent reaction. Involved in DNA non-homologous end joining (NHEJ) required for double-strand break repair and V(D)J recombination.
LTBP3	Latent transforming growth factor-beta-binding protein 3; associated with Dental anomalies and short stature
MACROD2	Macro domain-containing 2; Probably required for the association of ORC on chromatin during G1 to establish pre-replication complex (preRC) and to heterochromatic sites in post-replicated cells. Mutations cause Kabuki syndrome.
MAP2K1	Mitogen-activated protein kinase kinase; Growth factor signalling pathway.
MASP1	Mannan-binding lectin serine protease 1; Mutations cause 3MC syndrome: the main features of these syndromes are facial dysmorphism, cleft lip and palate, postnatal growth deficiency, cognitive impairment, and hearing loss.
MCM10	Minichromosome maintenance complex component 10; MCM10 mRNA level increased at the G1/S boundary when quiescent normal human fibroblasts were induced to proliferate with serum. MCM10 associated with nuclease-resistant nuclear structures throughout S phase and dissociated from them in G2 phase. MCM10 associated with ORC2 (ORC2L; 601182) when overexpressed in COS-1 cells, and it interacted with ORC2, MCM2 (116945), and MCM6 (601806) in a yeast 2-hybrid system.
MCM2	Minichromosome maintenance complex component 2; The MCM2-7 complex is comprised of 6 subunits, MCM2 through MCM7, and is a ring-shaped heterohexameric ATPase involved in DNA replication
MCM3	Minichromosome maintenance complex component 3; The MCM2-7 complex

	is comprised of 6 subunits, MCM2 through MCM7, and is a ring-shaped heterohexameric ATPase involved in DNA replication.
MCM4	Minichromosome maintenance complex component 4; The MCM2-7 complex is comprised of 6 subunits, MCM2 through MCM7, and is a ring-shaped heterohexameric ATPase involved in DNA replication.
MCM5	Minichromosome maintenance complex component 5; The MCM2-7 complex is comprised of 6 subunits, MCM2 through MCM7, and is a ring-shaped heterohexameric ATPase involved in DNA replication.
MCM6	Minichromosome maintenance complex component 6; The MCM2-7 complex is comprised of 6 subunits, MCM2 through MCM7, and is a ring-shaped heterohexameric ATPase involved in DNA replication.
MCM7	Minichromosome maintenance complex component 7; The MCM2-7 complex is comprised of 6 subunits, MCM2 through MCM7, and is a ring-shaped heterohexameric ATPase involved in DNA replication.
MCM8	Minichromosome maintenance complex component 8; Absence of MCM8 in human U2OS cells reduced growth and homologous recombination (HR) efficiency under conditions of replication stress.
MDC1	Mediator of DNA-damage checkpoint 1. Mdc1 -/- mice were born at the expected mendelian frequency, but they showed a phenotype similar to that of H2ax -/- mice, including growth retardation, male infertility, immune defects, chromosome instability, DNA repair defects, and radiation sensitivity
MLL2	Myeloid/lymphoid or mixed lineage leukemia 2; Kabuki syndrome
MMP14	Matrix metalloproteinase 14; Winchester syndrome - syndrome characterized by short stature, severe joint contractures, peripheral corneal opacities, coarsened facies, dissolution of carpal and tarsal bones, and generalized osteoporosis.
NBAS	Neuroblastoma-amplified sequence; Short stature, optic nerve atrophy, and Pelger-Huet anomaly.
NDUFAF2	NADH dehydrogenase (ubiquinone) complex i, assembly factor 2 mitochondrial complex deficiency 1; Associated with a number of clinical features including intrauterine growth retardation, marked growth and developmental delay in humans.
NDUFAF3	NADH dehydrogenase (ubiquinone) complex i, assembly factor 3; Associated with a number of clinical features including intrauterine growth retardation, marked growth and developmental delay in humans.
NDUFAF4	NADH dehydrogenase (ubiquinone) complex i, assembly factor 4; mitochondrial complex deficiency 1. Associated with a number of clinical features including intrauterine growth retardation, marked growth and developmental delay in humans.
NDUFB3	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3, 12kDa; mitochondrial complex deficiency 1. Associated with a number of clinical features including intrauterine growth retardation, marked growth and developmental delay in humans.
NDUFB9	NADH-ubiquinone oxidoreductase 1 beta subcomplex, 9. Associated with a number of clinical features including intrauterine growth retardation, marked growth and developmental delay in humans.
NDUFS1	NADH-ubiquinone oxidoreductase Fe-S protein 1; Associated with a number

	of clinical features including intrauterine growth retardation, marked growth and developmental delay in humans.
NDUFS2	NADH-ubiquinone oxidoreductase Fe-S protein 2; Associated with a number of clinical features including intrauterine growth retardation, marked growth and developmental delay in humans.
NDUFS4	NADH-ubiquinone oxidoreductase Fe-S protein 4; mitochondrial complex deficiency 1. Associated with a number of clinical features including intrauterine growth retardation, marked growth and developmental delay in humans. Leigh syndrome.
NDUFS6	NADH-ubiquinone oxidoreductase Fe-S protein 6; Associated with a number of clinical features including intrauterine growth retardation, marked growth and developmental delay in humans.
NF1	Neurofibromin 1; Stimulates the GTPase activity of Ras. NF1 shows greater affinity for Ras GAP, but lower specific activity. May be a regulator of Ras activity. Macrocephaly and short stature have been reported in several clinical studies of NF1.
NHEJ1	Nonhomologous end-joining factor 1; defects are known to cause severe combined immunodeficiency with microcephaly, growth retardation, and sensitivity to ionizing radiation.
NIPBL	Nipped-b, drosophila, homolog of; Cornelia de Lange syndrome 1
NPR2	Natriuretic peptide receptor 2; defects are associated with short stature with nonspecific skeletal abnormalities.
NUBPL	Nucleotide-binding protein-like protein; mitochondrial complex deficiency 1. Associated with a number of clinical features including intrauterine growth retardation, marked growth and developmental delay in humans
ORC1	Origin recognition complex, subunit 1, s. Cerevisiae, homolog of; *ORC1*, a subunit of the origin recognition complex, is a key component of the DNA replication licensing machinery that also plays a role in controlling centriole and centrosome copy number in human cells independent of its role in DNA replication. Mutations cause Meier-Gorlin syndrome 1 -almost all cases have primordial dwarfism with substantial prenatal and postnatal growth retardation.
OTX2	Orthodenticle, drosophila, homolog of, 2; *OTX2* defects have been described to cause short stature and developmental delay.
PAPPA	Pregnancy-associated plasma protein A, pappalysin 1; Metalloproteinase which specifically cleaves IGFBP-4 and IGFBP-5, resulting in release of bound IGF. Cleavage of IGFBP-4 is dramatically enhanced by the presence of IGF, whereas cleavage of IGFBP-5 is slightly inhibited by the presence of IGF.
PCNT	Pericentrin; Microcephalic osteodysplastic primordial dwarfism, type II.
PDCD4	Programmed cell death 4; It's been proposed that regulated degradation of PDCD4 in response to mitogens allows efficient protein synthesis and consequently cell growth.
PEG1	Paternally expressed gene 1; The *PEG1* gene maps to an imprinted region of mouse chromosome 6 and is expressed monoallelically from the paternal allele. When the null allele is paternally transmitted, the offspring exhibits severe intrauterine growth retardation.

PEG3	Paternally expressed gene 3; The heterozygous mice that inherited the mutant allele from the paternal germline were smaller
PEX2	Peroxisomal biogenesis factor 2; Somewhat implicated in the biogenesis of peroxisomes. Drosophila pex mutants, including *PEX2*, faithfully recapitulated several key features of human peroxisome biogenesis disorder, including impaired peroxisomal protein import, elevated very long chain fatty acid (VLCFA) levels, and growth retardation
PFKM	Phosphofructokinase, muscle type; catalyzes the third step of glycolysis, the phosphorylation of fructose-6-phosphate (F6P) by ATP to generate fructose-1,6-bisphosphate (FBP) and ADP. Thought to regulate growth and metabolism
PHKA1	Phosphorylase kinase, alpha 1 (muscle); Phosphorylase b kinase catalyzes the phosphorylation of serine in certain substrates, including troponin I.Defects cause glycogen storage disease.
PHKA2	Phosphorylase kinase, alpha 2 (liver); Phosphorylase b kinase catalyzes the phosphorylation of serine in certain substrates, including troponin I. Defects cause glycogen storage disease.
PHKB	Phosphorylase kinase, beta; Phosphorylase b kinase catalyzes the phosphorylation of serine in certain substrates, including troponin I. The beta chain acts as a regulatory unit and modulates the activity of the holoenzyme in response to phosphorylation. Defects cause glycogen storage disease.
PHKG2	Phosphorylase kinase, testis/liver, gamma-2; known to cause growth retardation and glycogen storage disease.
PIK3CA	Phosphatidylinositol 3-kinase, catalytic, alpha, severe growth failure described in humans.
PITX2	Paired-like homeodomain transcription factor 2; transcription factor PITX2 is rapidly induced by the WNT/DVL/beta-catenin pathway and is required for effective cell type-specific proliferation by directly activating specific growth-regulating genes.
PLAGL1	Pleomorphic adenoma gene-like 1; *PLAGL1* knockdown in mice resulted in intrauterine growth restriction, altered bone formation, and neonatal lethality
PLK4	Polo-like kinase 4; Serine/threonine-protein kinase that plays a central role in centriole duplication. Defects cause growth retardation with dwarfism (up to -8 SD).
POC1A	POC1 centriolar protein, chlamydomonas, homolog of, a; short stature, onychodysplasia, facial dysmorphism, and hypotrichosis;
POLE	Polymerase, dna, epsilon; facial dysmorphism, immunodeficiency, livedo, and short stature.
POU1F1	POU class 1 homeobox 1; Transcription factor involved in the specification of the lactotrope, somatotrope, and thyrotrope phenotypes in the developing anterior pituitary. Activates growth hormone and prolactin genes.
PPP1R15B	Protein phosphatase 1, regulatory subunit 15B; Microcephaly, short stature, and impaired glucose metabolism 2.
PRKAG2	AMP-activated protein kinase, noncatalytic, gamma-2; glycogen storage disease.

PROP1	PROP paired-like homeobox 1; affected mice are of normal body size at birth but postnatal growth is severely retarded and the body size of adult animals is approximately one-third of normal.
PYGL	Glycogen phosphorylase, liver; short stature and glycogen storage disease.
PYGM	Glycogen phosphorylase, muscle; glycogen storage disease.
RAB3IP	RAB3A interacting protein (rabin3). Variants in RABIP have been reported in a patient with short stature.
RIEG1	Rieg bicoid-related homeobox transcription factor 1; transcription factor RIEG1 is rapidly induced by the WNT/DVL /beta-catenin pathway and is required for effective cell type-specific proliferation by directly activating specific growth-regulating genes.
RIN1	Ras and Rab interactor 1; Ras effector protein. Can affect RAS signalling at different levels. First, by competing with RAF1 protein for binding to activated Ras. Second, by enhancing signaling from ABL1 and ABL2, which regulate cytoskeletal remodelling.
RNU4ATAC	RNA, U4ATAC small nuclear; Microcephalic osteodysplastic primordial dwarfism, type I.
RSPRY1	Ring finger- and spry domain-containing protein 1; Spondylopeimetaphyseal dsyplasia, Faden-Alkuraya type. Phenotype: progressive spondyloepimetaphyseal dysplasia, short stature, facial dysmorphism, short fourth metatarsals, and intellectual disability.
RTTN	Rotatin; Microcephaly, short stature, and polymicrogyria with seizures
SEMA3E	Semaphorin 3E; suspected to cause CHARGE syndrome (short stature is a feature).
SF3B4	Splicing factor 3b, subunit 4; Nager syndrome is the prototype for a group of disorders collectively referred to as the acrofacial dysostoses (AFDs), which are characterized by malformation of the craniofacial skeleton and the limbs.
SHOC2	SOC-2 suppressor of clear homolog (C. elegans); Regulatory subunit of protein phosphatase 1 (PP1c) that acts as a M-Ras/MRAS effector and participates in MAPK pathway activation. Noonan-like syndrome with associated growth restriction.
SHOX	Short stature homeobox; Controls fundamental aspects of growth and development.
SLC37A4	Solute carrier family 26, member 4; Sodium-independent transporter of chloride and iodide. Glycogen storage disease.
SMC1A	Structural maintenance of chromosomes 1A; Involved in chromosome cohesion during cell cycle and in DNA repair. Cornelia de Lange syndrome.
SOCS2	Suppressor of cytokine signaling 2; SOCS family proteins form part of a classical negative feedback system that regulates cytokine signal transduction. SOCS2 appears to be a negative regulator in the growth hormone/IGF1 signaling pathway.
SRCAP	SNF2-related CREBBP activator protein; Catalytic component of the SRCAP complex which mediates the ATP-dependent exchange of histone H2AZ/H2B dimers for nucleosomal H2A/H2B, leading to transcriptional regulation of selected genes by chromatin remodeling. Defects causeFloating-Harbor syndrome, a rare genetic disorder characterized by proportionate short stature, delayed bone age, delayed speech development,

	and typical facial features.
TIMP1	TIMP metallopeptidase inhibitor 1; Complexes with metalloproteinases (such as collagenases) and irreversibly inactivates them by binding to their catalytic zinc cofactor. Also mediates erythropoiesis in vitro; but, unlike IL-3, it is species-specific, stimulating the growth and differentiation of only human and murine erythroid progenitors. Known to act on MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-11, MMP-12, MMP-13 and MMP-16
TMEM126B	Transmembrane protein 126B. mitochondrial complex deficiency 1. Associated with a number of clinical features including intrauterine growth retardation, marked growth and developmental delay in humans
TRIM37	tripartite motif containing 37; E3 ubiquitin-protein ligase. Defects cause Mulibrey nanism.
TRMT10A	tRNA methyltransferase 10, S. cerevisiae, homolog of, A; Microcephaly, short stature, and impaired glucose metabolism 1.
UBR1	Ubiquitin protein ligase E3 component n-recognin 1; E3 ubiquitin-protein ligase which is a component of the N-end rule pathway. Binds leucine and is a negative regulator of the leucine-mTOR signaling pathway, thereby controlling cell growth. Defects are associated with Johanson-Blizzard syndrome is an autosomal recessive disorder characterized by poor growth, mental retardation, and variable dysmorphic features, including aplasia or hypoplasia of the nasal alae, abnormal hair patterns or scalp defects, and oligodontia.

153 biological candidate genes which are associated with syndromic growth disorders, skeletal dysplasias, growth plate biology, cell proliferation, DNA replication and repair, code for proteins that interact with partner proteins known to be involved in growth pathways or shown to affect growth in mouse models but without proven link to human linear growth.

Supplementary Table 2. Clinical, biochemical and genetic features in individuals with Silver–Russell syndrome (SRS)

Pt no.	Birth weight SDS	Height SDS	BMI SDS	Target Height SDS	GH basal (µg/L)	GH max (µg/L)	IGF-1 (ng/ml)	IGF-1 SDS	Clinical features	Genetic variants
38	-1.8	-3.7	+0.5	+0.8	12.6	N/D	3.3	-2.8	Classical	H19 hypomethyl-ation
39	-2.3	-4.3	-4.9	-0.5	4.6	12.5	28	-3.4	Classical, blue sclera, high-pitched voice,small face	MatUPD7

H19 hypomethylation, hypomethylation of the imprinting control region 1 (IGF2/H19) in chromosome 11q15; MatUPD7, maternal uniparental disomy of chromosome 7; + parents consanguineous; -, parents not consanguineous. Both patients were previously reported in Storr *et al,* 2015 [10].

Supplementary Table 3. Genetic variants identified in the GH insensitivity patients by unbiased Analysis 3 (109 variants in 77 candidate genes; n=43 patients)

Gene	No. variants	No. patients	Gene	No. Variants	No. Patients
ANKRD30A	1	2	*MICA*	2	4
ANTXR2	1	2	*MTCH2*	2	3
*AQP7**	1	2	*MUC17*	1	2
ARSD	2	4	*MUC2*	2	6
BHLHE22	2	3	*MUC5AC*	1	3
C11orf40	1	3	*MUC6**	2	4
C11orf80	1	2	*MYO15B*	1	3
CAMKK2	1	2	*NKAP*	1	2
CCDC66	2	4	*NLRC5*	1	3
CD200R1	1	2	*NOTCH4*	1	3
CD6	1	3	*NPIPB8*	1	3
CDC42EP1	1	2	*OR2T2/OR2T35*	1	2
CTBS	1	2	*OR4A16*	4	2
DFNB59	1	2	*OR51B6*	1	2
DSPP	2	4	*OTOP1*	1	2
EI24	1	2	*PABPC1*	2	4
FAM104B	1	2	*PABPC3*	1	2
FAM174B	1	2	*PODXL*	1	3
FAM188B	1	2	*PRKRA*	1	2
FBXL21	1	3	*PRSS3*	1	2
FNDC1	1	2	*PSG8*	1	2
FRG2/FRG2B	1	3	*RPL14*	1	3
GOLGA6L2	4	5	*RXFP2*	1	3
GRIA3	1	2	*SLC25A5*	2	2
GXYLT1	3	4	*SLX4*	1	3
HGC6.3	1	2	*SRRM3*	1	3
*HLA-DRB1**	4	7	*TAS2R19*	1	3
IGSF3	1	2	*TAS2R31**	1	3
JPH3	1	3	*TAS2R43*	3	3
KIAA0040	1	2	*TEKT4*	1	2
KRTAP4-5	1	2	*UBXN11*	1	2
LCE4A	1	2	*USF3*	1	2
LGALS8	1	3	*ZAN*	1	2
LILRA3	1	2	*ZFPM1*	1	2
LILRB1	1	2	*ZNF534*	2	3
LOC100129697	2	5	*ZNF598*	1	2
LOR	1	2	*ZNF717*	6	5
MAFA	1	3	*ZNF91*	2	2
MAP3K4	2	2			

Genes were selected if they contained variants which satisfied the following criteria: call quality at least 20, read depth at least 10, allele frequency 0.1% or less in any of the 1000 genomes, ExAC, and all of the NHLBI exomes. Data selected was outside 0.1% of most exonically variable 100 base windows in healthy public genomes and outside 0.1% most exonically variable genes in healthy public genomes (1000 genomes, ExAC). Predicted deleterious changes were defined as disease associated if according to computed ACMG guidelines were classified as pathogenic or likely pathogenic or associated with loss of function of a gene being frameshift, in-frame indel, or start/stop codon change, missense or splice-site change up to 2 bases into the intron. Homozygous or double heterozygous changes were selected if present in novel genes in at least 2 patients but not in the controls. Variants were excluded if the same variant occurred in four or more patients in the likelihood that these were not causal. * Genes affected in both GH and IGF-1 groups.

Supplementary Table 4. Univariate logistic regression analysis of age, sex and consanguinity as predictor for a positive genetic diagnosis

Variable	p value	Odds Ratio	95% CI Lower Limit	95% CI Upper Limit
Age	0.057	0.92	0.84	1.003
Sex	0.25	1.59	0.72	3.48
Consanguinity	**0.0001**	18.29	6.56	51.06

CI, confidence intervals.