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

Background: 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 were 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.

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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.

Clinical Study	L Shapiro and others	Genetic diagnosis of GH insensitivity	177:6	486

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 characterised by intrauterine and/or post-natal 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 (Fig. 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

Figure 1

Summary of candidate gene (CGS) and whole-exome sequencing (WES) in the GH and IGF-1 insensitivity patients. (A) 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. (B) Whole-exome sequencing (WES) data analyses: number of patients assessed and variants identified.

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: $7\text{--}1195\text{ }\mu\text{g/L}$. 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) and 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 (Fig. 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>). Two 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 (Fig. 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 (2×100) sequencing on an Illumina HiSeq 2000 at Otogenetics (Norcross, GA, USA). 31 patients and 8 relatives were sequenced using SureSelect Human All Exon v5 (51Mb) capture and paired-end (2×100) sequencing on an Illumina 2500 Standard run (minimum coverage $50\times$) at Oxford Gene Technology (OGT, Oxford, UK). $>90\%$ of target bases were covered $10\times$. For comparison, WES data from 43 in-house controls generated on the same platforms were analysed 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

Clinical Study	L Shapiro and others	Genetic diagnosis of GH insensitivity	177:6	488

resulting VCF files were uploaded to Ingenuity Variant Analysis (Qiagen; <http://www.ingenuity.com/>). 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 (<http://exac.broadinstitute.org>)) were included. Common variants were filtered out by excluding those with an allele frequency of $\geq 0.1\%$ 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 (<http://sift.jcvi.org>) or PolyPhen (<http://genetics.bwh.harvard.edu/pph2/>) were explored further (Fig. 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.

Analysis 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 (Supplementary Table 1, see section on supplementary data given at the end of this article). An autosomal recessive model was adopted i.e. homozygous, hemizygous (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 hypothesised 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.

Analysis 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 mentioned previously, 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 (www.mutationtaster.org) and Human Splicing Finder (HSF, version 3.0; <http://www.umd.be/HSF3/>) to predict the functional outcome. VEP (http://grch37.ensembl.org/Homo_sapiens/Tools/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 (<https://www.ncbi.nlm.nih.gov/pubmed/>), OMIM (<http://www.omim.org/>) and String (<http://string-db.org>) 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).

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) (Figs 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: $40.7 \mu\text{g/L}$ (range: 1.8 – 398 , $n=21$) and $123.2 \mu\text{g/L}$ (range: 15.7 – 1195 , $n=20$). All had homozygous variants in *GHR* with the exception of 2 patients (21 and 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: $119 \mu\text{g/L}$) 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

Three GHI patients (28, 29, 30) had homozygous *IGFALS* variants (mean serum IGF1 SDS -2.7 (-3.6 to -1.9) and mean peak GH concentration $20.5 \mu\text{g/L}$ (16.0 – $28.9 \mu\text{g/L}$). 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 and 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 $17.5 \mu\text{g/L}$) (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) (Fig. 1A and Supplementary Table 2) (10).

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

Patient number	Age (year)	Sex	Consanguinity/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
1	8.0	M	+/Pakistani	−0.5	−4.0	0.7	−0.9	13.2	119.0	18.2	−2.6
2 ^s	4.2	F	+/Pakistani	0.1	−4.2	−1.0	0.7	16.3	33.3	<22.4	−2.5 ^a
3 ^s	7.5	M	+/Pakistani	−2.9	−4.5	−1.2	−1.3	4.0	>33	1.4	−2.8
4	7.7	M	+/Indian	−1.7	−3.1	−2.4	−1.9	3.2	30.3	11.2	−2.6
5 ^b	14.7	M	+/Pakistani	0.7	−3.0	−0.7	−1.0	11.3	39.6	9.1	−3.1
6 ^b	2.3	M	+/Pakistani	NK	−4.7	−0.5	−1.0	50.8	46.0	<22.4	−3.1 ^a
7	2.4	F	+/Pakistani	−1.8	−5.0	−0.4	N/D	3.4	26.7	134.3	−2.3
8	6.8	F	+/Pakistani	−0.3	−4.1	−0.2	−0.9	56.1	30.3	30.3	−4.0
9 ^b	2.4	M	−/Argentinian	0.2	−7.7	−0.8	−1.9	NK	57.0	UD	−2.5 ^a
10 ^b	14.3	M	−/Argentinian	0.5	−8.4	0.0	−1.9	NK	88.0	9.0	NK
11	8.4	M	+ /Turkish	−6.0	−8.7	1.8	−1.9	NK	79.0	5.1	NK
12	5.8	M	+ /NK	−0.7	−7.7	−0.7	−1.4	NK	3344344A32.5	UD	−2.5 ^a
13	NK	M	NK/Mexican	NK	NK	NK	NK	NK	NK	NK	NK
14	4.2	M	+ /Bangladeshi	1.6	−6.9	−5.6	−1.8	398.0	1195.0	<25	−2.5 ^a
15	1.8	F	+ /Bangladeshi	−0.2	−5.7	−2.2	−2.3	36.0	ND	<25	−2.5 ^a
16	1.5	F	+ /Bangladeshi	−1.2	−6.1	0.7	−2.0	60.0	ND	<25	−2.5 ^a
17	1.1	M	+ /Kuwaiti	2.6	−5.0	0.8	−0.3	>47	>47	<30	−2.5 ^a
18	5.7	M	+ /Kuwaiti	−0.6	−5.3	1.7	−1.3	14.4	35.0	32.9	−2.5 ^a
19 ^c	9.4	M	+ /Egyptian	2.1	−6.4	−5.5	−1.5	16.0	33.3	9.5	−8.5
20 ^c	6.2	M	+ /Egyptian	2.1	−5.5	−2.8	−1.5	1.8	15.7	8.1	−8.2
21	10.5	M	− /Caucasian	1.5	−2.9	1.8	1.0	42.2	ND	42.0	−3.0
22 ^d	15.3	F	− /Thai	−2.3	−9.1	−1.3	−2.15	ND	41	67.5	−2.5
23 ^d	5.8	F	− /Thai	−1.8	−6.9	−1.0	−2.15	15.9	108.44	<1	−2.8 ^a
24 ^e	4.0	M	+ /Indian	−1.3	−7.9	−1.3	−2.62	12.4	ND	<25	−3.1 ^a
25 ^e	1.3	M	+ /Indian	−2.6	−8.9	−2.0	−2.62	24.5	ND	ND	ND

Clinical Study	L Shapiro and others	Genetic diagnosis of GH insensitivity	177:6	491

Clinical features	Genetic variants			MAF ExAC	Predicted outcome (novel variants)	Reference	No. genes analysed by CGS
	<i>GHR</i> gene	<i>IGFALS</i> gene	3M syndrome genes				
No	Hom c.618+792A>G, p.Met206_ Met207ins36			0	–	(26)	1 (<i>GHR</i>)
Classical	Hom c.618+792A>G, p.Met206_ Met207ins36			0	–	(26)	1 (<i>GHR</i>)
Classical	Hom c.618+792A>G, p.Met206_ Met207ins36			0	–	(26)	1 (<i>GHR</i>)
Classical	Hom c.618+792A>G, p.Met206_ Met207ins36			0	–	(26)	1 (<i>GHR</i>)
Classical	Hom c.618+792A>G, p.Met206_ Met207ins36			0	–	(26)	1 (<i>GHR</i>)
Classical	Hom c.618+792A>G, p.Met206_ Met207ins36			0	–	(26)	1 (<i>GHR</i>)
Classical	Hom c.618+792A>G, p.Met206_ Met207ins36			0	–	(26)	1 (<i>GHR</i>)
Classical	Hom c.618+792A>G, p.Met206_ Met207ins36			0	–	(26)	1 (<i>GHR</i>)
Classical	Hom c.618+792A>G, p.Met206_ Met207ins36			0	–	(26)	1 (<i>GHR</i>)
Classical	Hom c.198C>A, p.Cys66*			0	High impact (VEP)	Unpublished	1 (<i>GHR</i>)
Classical	Hom c.198C>A, p.Cys66*			0	High impact (VEP)	Unpublished	1 (<i>GHR</i>)
Classical	Hom c.700C>T, p.Gln234*			0	High impact (VEP)	Unpublished	1 (<i>GHR</i>)
Classical	Hom c.740T>C, p.Leu247Pro (p.L229P)			0	Deleterious (SIFT score 0)	(35)	1 (<i>GHR</i>)
Classical	Hom c.594A>G, p.Glu198* (E180X)			0	–	(36)	1 (<i>GHR</i>)
Classical	Hom c.785-6 T>A, p.Asp264Glyfs*5 (p.D244GfsX5)			0	–	(37)	1 (<i>GHR</i>)
Classical	Hom c.247C>T, p.Gln83* (Q65X)			0	–	(38)	1 (<i>GHR</i>)
Classical	Hom c.703C>T, p.Arg235* (R217X)			8.2 × E ⁻⁶ Y	–	(39)	1 (<i>GHR</i>)
Classical	Hom c.703C>T, p.Arg235* (R217X)			8.2 × E ⁻⁶ Y	–	(39)	1 (<i>GHR</i>)
Classical	Hom c.703C>T, p.Arg235* (R217X)			8.2 × E ⁻⁶ Y	–	(39)	1 (<i>GHR</i>)
Classical	Hom c.439+1 G>A, p.Arg89Serfs*47			0	–	(10)	1 (<i>GHR</i>)
Classical	Hom c.439+1 G>A, p.Arg89Serfs*47			0	–	(10)	1 (<i>GHR</i>)
Classical	c.266+83G>T, p.? c.723C>T, p.Gly241_ Glu261del (p.G223_ E243del)			0	–	(40)	1 (<i>GHR</i>)
Classical	Hom. c.723C>T, p.Gly241_Glu261del (p.G223_E243del)			0	–	(41)	1 (<i>GHR</i>)
Classical	Hom. c.723C>T, p.Gly241_Glu261del (p.G223_E243del)			0	–	(41)	1 (<i>GHR</i>)
Classical	Hom c.599A>G, p.Asn200Ser			0	Deleterious (SIFT)	Unpublished	1 (<i>GHR</i>)
Classical	Hom c.599A>G, p.Asn200Ser			0	Deleterious (SIFT)	Unpublished	1 (<i>GHR</i>)

(Continued)

Clinical Study	L Shapiro and others	Genetic diagnosis of GH insensitivity	177:6	492

Table 1 Continued.

Patient number	Age (year)	Sex	Consanguinity/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
26	1.6	F	–/Caucasian	–1.4	–4.3	–1.4	NK	25.5	ND	<25	–2.5 ^a
27	3.1	F	+/Arabic-Syrian	–1.1	–4.5	–1.2	–1.02	3.3	75.0	8.0	–7.3
28 ^a	8.0	F	+/Indian	–3.0	–3.9	–2.5	–2.32	2.2	28.9	<16.0	–2.5 ^a
29 ^a	6.1	M	+/Indian	–2.1	–2.0	NK	–4.1	1.1	16.7	25.9	–1.9
30	13.5	F	+/Moroccan	–1.3	–3.3	–1.75	–1.63	9.7	16.01	66.0	–3.6
31	4.6	M	+/Bedouin	–3.2	–7.4	–0.7	–0.5	6.0	32	6.4	–2.5
32	1.0	M	+/Kuwaiti	–1.6	–6.4	–2.3	–1.3	2.1	18.2	30.5	–2.5
33	3.0	F	+/Kuwaiti	–5.2	–5.7	–4.7	NK	9.1	15	<3.0	–2.7 ^a
34 ^a	1.1	F	+/Kuwaiti	–3.8	–4.9	–0.4	–0.34	4.2	37.2	ND	ND
35 ^a	0.1	F	+/Kuwaiti	–2.6	–5.1	0.7	–0.34	5.4	10.8	105.0	–0.2
36	0.06	F	+/Kuwaiti	–1.5	–4.5	0.5	–1.2	41.0	33	<27	–2.6
37	10.8	F	+/Bangladeshi	–2.9	–6.8	1.0	–2.39	NK	6.5	159.0	–0.3
38	6.6	F	–/Caucasian	–2.7	–3.1	–1.3	–0.46	17.5	9.6	367.0	2.0

Diagnosis by whole-exome sequencing (WES)

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 9849 genes (Fig. 1B).

Analysis 1

Figure 2 and Table 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 *SOS1* ($n=1$)).

Clinical Study	L Shapiro and others	Genetic diagnosis of GH insensitivity	177:6	493

Clinical features	Genetic variants			MAF ExAC	Predicted outcome (novel variants)	Reference	No. genes analysed by CGS
	<i>GHR</i> gene	<i>IGFALS</i> gene	3M syndrome genes				
Classical	c.168C>A, p.Cys56* (p.C38X) c.922G>A, p.Gly308Arg			8.2 × E ⁻⁶ ^Y	–	(42)	1 (<i>GHR</i>)
Classical	Hom c.344A>C, p.Asn115Thr			0	Deleterious (SIFT score 0)	Unpublished	1 (<i>GHR</i>)
No		Hom c.401T>A p.Leu134Gln (p.L134Q)			–	(43)	2 (<i>GHR</i> , <i>IGFALS</i>)
No		Hom c.401T>A p.Leu134Gln (p.L134Q)			–	(43)	1 (<i>IGFALS</i>)
No		Hom c.1291delT, p.Trp431Glyfs*11 (p.W431Gfs11)		0	–	(10)	2 (<i>GHR</i> , <i>IGFALS</i>)
Classical			<i>OBSL1</i> Hom c.1463C>T, p.Arg489* (p.R489X)	0	–	(23)	2 (<i>IGF1R</i> , <i>OBSL1</i>)
Classical Hypermobility, prominent heels			<i>OBSL1</i> Hom c.1463C>T p.Arg489* (p.R489X)	0	–	(23)	4 (<i>GHR</i> , <i>IGFALS</i> , <i>IGF1</i> , <i>OBSL1</i>)
Classical Hypermobility, prominent heels			<i>OBSL1</i> Hom c.1359insA, p.Glu454Argfs*11 (p.E454RfsX11)	0	–	(23)	2 (<i>IGF1</i> , <i>OBSL1</i>)
Classical. Hypermobility			<i>OBSL1</i> Hom c.1359insA, p.Glu454Argfs*11 (p.E454RfsX11)	0	–	(23)	2 (<i>IGF1</i> , <i>OBSL1</i>)
Classical Prominent heels			<i>OBSL1</i> Hom c.1359insA, p.Glu454Argfs*11 (p.E454RfsX11)	0	–	(23)	2 (<i>IGF1R</i> , <i>OBSL1</i>)
Classical hypermobility, prominent heels short fingers, trident hands, short rib cage, bilateral DDH	<i>OBSL1</i> Hom c.1359insA, p.E454Rfs*11 (p.E454RfsX11)			0	–	(23)	4 (<i>GHR</i> , <i>IGFALS</i> , <i>IGF1</i> , <i>OBSL1</i>)
Disproportion large head, short limbs, lumbar lordosis	<i>CUL7</i> Hom c.2710C>T, p.Arg904*			0	–	(44)	3 (<i>IGF1R</i> , <i>OBSL1</i> , <i>CUL7</i>)
Triangular face, long fingers	<i>IGF1R</i> gene Het c.112G>A, p.Asp38Asn (p.D38N)			0	–	(10)	1 (<i>IGF1R</i>)

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 (6^Y); ins, insertion; fs, frameshift; *, termination site; as, acceptor site; ds, donor site; X, stop codon; del, deletion; ^Y, 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; ^Y, 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 (<http://varnomen.hgvs.org/>) guidelines. Italicised patient numbers indicate those patients previously reported in Storr *et al.* 2015 (10).

Figure 2

Genetic diagnoses in the GH and IGF-1 insensitivity patients.

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

Clinical Study	L Shapiro and others	Genetic diagnosis of GH insensitivity	177:6	495

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 ([Supplementary 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 ; $P<0.0001$) ([Fig. 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* $\delta\Psi$ 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 ([Supplementary 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 $\geq 7.0\mu\text{g/L}$) 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 *GHI*, *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

Clinical Study	L Shapiro and others	Genetic diagnosis of GH insensitivity	177:6	496

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

Patient number	Age (year)	Sex	Consanguinity/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
41	6.9	M	+/Kuwaiti	0.3	−2.1	−2.7	−1.5	1.1	>32	35.4	−2.3
42	8.9	F	−/Polish	−2.1	−3.2	−1.6	0.6	21.7	10.5	47	−2.4
43	13.1	M	−/Mexican–Russian	−3.0	−3.8	−1.5	−1.5	0.4	26.6	7.4	−2.63
44	1.9	F	+/Pakistani	−3.5	−5.7	−1.6	N/D	3.0	5.0	7.5	−1.8
45	0.3	F	+/Kuwaiti	−5.8	−5.5	−0.6	NK	22.5	26.7	116	−1.1
46	15.4	F	+/Pakistani	−3.4	−5.0	1.0	−3.4	0.1	13	35	−2.5
47 ^h	3.5	F	+/Kuwaiti	−0.82	−3.9	−1.2	−1.8	9.5	85	<30	−5.3
48 ^h	2.2	F	+/Kuwaiti	−0.34	−2.5	−0.4	1.8	22	ND	<30	−5.3
49	2.0	F	+/Kuwaiti	−2.1	−6.7	−2.3	0.3	35	>35	<30	−5.3
50	1.2	F	+/Kuwaiti	+0.12	−6.7	−1.3	−1.4	11.3	>32	<10	−2.1
51	1.4	M	+/Kuwaiti	+1.99	−5.9	−0.2	−0.1	>35	>35	<30	−5.3

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).

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, 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

Clinical Study	L Shapiro and others	Genetic diagnosis of GH insensitivity	177:6	497

Clinical features	Genetic variants				MAFExAC	Predicted outcome (novel variants)	Reference	No. genes analysed by CGS
	Noonan syndrome genes	3M syndrome genes	IGFALS gene	GHR gene				
Low set ears, undescended left testis	PTPN11 Het c.417G>C p.Glu139Asp (p.E139D)				0	–	(25)	1 (<i>GHR</i>)
Low set ears, hypertelorism, mild ptosis, low posterior hairline	PTPN11 Het c.853T>C, p.Phe285Leu (p.F285L)				0	–	(25)	1 (<i>GHR</i>)
No	SOS1 Het c.3418T>A p.Leu1140Ile				3.5 × E ⁻⁵ ‡	Disease causing (Mutation Taster)	Unpublished	2 (<i>GHR</i> , <i>IGFALS</i>)
Classical		CCDC8 Hom c.612dupG, p.Lys205Glufs*59 (p.Lys205GlufsX59)			1.8 × E ⁻⁵ ‡	–	(23)	1 (<i>GHR</i>)
Classical bilateral DDH		CUL7 Hom c.2988G>A, p.Trp996* (p.W996X)			0	–	(45)	1 (<i>GHR</i>)
Classical			c.1576G>A p.Asp526Asn c.632G>A , p.Trp211*		1.7 × E ⁻⁴ ‡	Deleterious (SIFT score 0)	Unpublished	1 (<i>GHR</i>)
					0	Deleterious (SIFT score 0)	Unpublished	
Classical				Hom c.70+4A>C , p.?	0	Variation predicted by HSF	Unpublished	1 (<i>GHR</i>)
Classical				Hom c.70+4A>C , p.?	0	Variation predicted by HSF	Unpublished	1 (<i>GHR</i>)
Classical				Hom c.70+4A>C , p.?	0	Variation predicted by HSF	Unpublished	1 (<i>GHR</i>)
Classical				Hom c.70+4A>C , p.?	0	Variation predicted by HSF	Unpublished	1 (<i>GHR</i>)
Classical				Hom c.70+4A>C , p.?	0	Variation predicted by HSF	Unpublished	1 (<i>GHR</i>)
Classical				Hom c.703C>T, p.Arg235* (R217X)	8.2 × E ⁻⁶ ‡	–	(39)	1 (<i>GHR</i>)

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; ‡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; ‡, 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.

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

Figure 3

Height SDS in patients with a genetic diagnosis and those with no genetic diagnosis. Diagnosed patients $n=50$, undiagnosed $n=55$; $*P=0.0001$.

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 *SOS1* gene variant was born SGA, had short stature and IGFD but no classical features of NS. The other patient with novel compound heterozygous *IGFALS* gene variants is shorter (-5.0 SDS) than most/all previously reported patients with *IGFALS* defects (1). This emphasises not only the importance of accurate clinical phenotyping prior to referral for genetic testing but also the difficulties in diagnosing many short stature syndromes. Noonan in particular, should be carefully considered when assessing a patient with features of GHI (31).

Eleven novel genetic variants were identified in *GHR*, *IGFALS* and *SOS1* genes. As functional studies were not undertaken on the novel variants, it remains a possibility that they are not responsible for the clinical phenotype. However, familial segregation and *in silico* prediction programmes have been utilised to substantiate them.

Table 3 Comparison of mean height SDS, IGF-1 SDS and peak GH levels (means $\pm$ S.D.) between individuals with genetic defects and those with no molecular diagnosis.

	No genetic diagnosis (Group 1)	GHR and GHR 6p mutations (Group 2)	3M gene mutations (Group 3)	Any genetic diagnosis (Group 4)	P value (95% CI)		
					Group 1 vs Group 2	Group 1 vs Group 3	Group 1 vs Group 4
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 ($1.3-2.6$)	<0.0001 ($1.2-2.8$)	<0.0001 ($0.89-2.1$)
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 ($0.87-2.45$)	0.3682 ($-1.6-0.6$)	0.0128 ($0.2-1.63$)
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 ($12.9-131.3$)	0.9676 ($-12.3-11.8$)	0.0594 ($-1.88-94.9$)

3M gene mutations, mutations identified in *CUL7*, *CCDC8* and *OBSL1*.

Except for cases 42 (compound heterozygous *IGFALS*) and 45 (heterozygous *SOS1*) the phenotypes are also typical for the identified genetic defects (1). Therefore, we are confident that these genetic variants explain the clinical presentation. According to ExAC, the *SOS1* gene is intolerant of loss of function variants (expected number of loss of function variants 57.5; observed loss of function variants 3, pLI=1.0) this increases the likelihood of this variant being pathogenic. The *IGFALS* variants are both predicted to be highly deleterious and the patient had reduced birth weight (SDS -3.4). Together these factors may contribute to the development of a more extreme phenotype. No dysmorphic features or other potential genetic variants in candidate genes were identified in this patient that could explain the more severe phenotype. However, we cannot rule out oligogenicity with a novel gene defect. Prenatal growth retardation in particular has been previously recognised to contribute to the heterogeneity of *IGFALS* defects (32).

We also identified a novel homozygous, predicted deleterious *FANCA* mutation in a patient with normal birth weight, short stature (-3.0 SDS) and IGFD (-2.0). Fanconi anaemia (FA), is an autosomal recessive trait, associated with skeletal and cardiac defects, pre- and post-natal growth retardation and malformation of the kidneys, although consistent with this case, 25% patients have no reported physical abnormalities (33). The mean age at presentation is typical (usually ~7 years) 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 other undiscovered growth genes exist which have not been 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 prioritise. Our data suggest 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.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EJE-17-0453>.

Declaration of interest

H S has received honoraria from Ipsen Pharmaceuticals and research grants from Ipsen and Sandoz Pharmaceuticals. MOS has a consultancy agreement with Ipsen Pharmaceuticals.

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Author contribution statement

L A M and L S performed bioinformatics analyses. S C, D G R, K M D and H L S contributed to patient recruitment, data collection and analysis. S C performed the phenotypic and statistical analyses. H L S wrote the manuscript with input from S C, L A M, L S and D G R.

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