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GHR gene transcript heterogeneity may explain phenotypic variability in *GHR* pseudoexon (6Ψ) patients

Sumana Chatterjee[1], Emily Cottrell[1], Stephen J. Rose[2], Talat Mushtaq[3], Avinaash V. Maharaj[1], Jack Williams[1], Martin O. Savage[1], Louise A. Metherell[1], Helen L. Storr[1]

[1]Centre for Endocrinology, William Harvey Research Institute, Barts and the London School of Medicine & Dentistry, Queen Mary University of London, London, UK.

[2]Birmingham Heartlands Hospital, Heart of England NHS Foundation Trust, Birmingham, UK.

[3]The Leeds Teaching Hospital NHS Trust, Leeds, UK.

Corresponding author:

Dr Helen Storr,

Reader and Honorary Consultant in Paediatric Endocrinology,

Centre for Endocrinology, John Vane Science Centre,

Charterhouse Square,

London EC1M 6BQ, UK.

Tel: +44 (0)20 7882 6198. Fax: +44 (0)20 7882 6197

E-mail: h.l.storr@qmul.ac.uk

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ABSTRACT:

Objectives: The homozygous GH receptor (*GHR*) pseudoexon (6Ψ) mutation leads to growth hormone insensitivity (GHI) with clinical and biochemical heterogeneity. We investigated whether transcript heterogeneity (6Ψ-*GHR* to WT-*GHR* transcript ratio) and/or concurrent defects in other short stature (SS) genes contribute to this.

Methods: 6Ψ-*GHR* and WT-*GHR* mRNA transcripts of 4 6Ψ patient (height SDS -4.2 to -3.1) and 1 control fibroblasts were investigated by RT-PCR. Transcripts were quantified by qRT-PCR and delta delta CT analysis and compared using ANOVA with Bonferroni correction. In eleven 6Ψ patients, 40 genes known to cause GHI/SS were analysed by targeted next generation sequencing.

Results: RT-PCR confirmed 6Ψ-*GHR* transcript in the 6Ψ patients but not control. 6Ψ-*GHR* transcript levels were comparable in patients 1 and 3 but significantly different among all other patients. The mean 6Ψ:WT transcript ratios ranged from 29-71:1 for patients 1-4 and correlated negatively with height SDS ($R=-0.85$; $p<0.001$). Eight deleterious variants in 6 genes were detected but the number of gene hits did not correlate with the degree of SS in individual 6Ψ patients.

Conclusion: Variable amounts of 6Ψ- and WT-*GHR* transcripts were identified in 6Ψ patients but no 6Ψ transcript was present in the control. Higher 6Ψ:WT *GHR* transcript ratio correlated with SS severity and may explain the phenotypic variability. Analysis of known SS genes suggested that phenotypic variation is independent of the genetic background. This is the first report of transcript

heterogeneity producing a spectrum of clinical phenotypes in different individuals harbouring an identical homozygous genetic mutation.

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INTRODUCTION:

Growth Hormone Insensitivity (GHI) is characterised by growth failure, IGF1 deficiency and normal or elevated GH levels. GHI encompasses a spectrum of genetic, phenotypic, and biochemical abnormalities associated with growth failure[1,2]. Monogenic defects of the GH-IGF1 axis leading to GHI have been identified in the *GHR*[3], *STAT5B*[4], *IGFALS*[5], *PAPPA2*[6] and *IGF1*[7] genes.

Splicing is the process by which introns are precisely identified and excised with the remaining exons united to form a translatable message[8]. Intronic DNA frequently encodes potential exonic sequences[9]. These "pseudoexons" are sequences between 50-300 nucleotides in length with apparently viable 5' and 3' splice sites[10,11]. Under normal circumstances, they are actively suppressed and not recognised by the splicing machinery[10,12-15]. However, point mutations in intronic DNA sequences can lead to the creation of new donor (5'), acceptor (3') splice or branch sites and activation of "pseudoexons". This often occurs within 100 nucleotide bases of the canonical splice site resulting in the inclusion of intronic sequences immediately flanking the exonic sequence[16]. Inclusion of intronic sequences by aberrant splicing is a recognised rare cause of several genetic diseases including neurofibromatosis type 1, cystic fibrosis, Duchenne and Becker muscular dystrophies[8,17].

The intronic growth hormone receptor *(GHR)* pseudoexon (6Ψ) mutation was first reported in 2001 in two sets of siblings from a highly consanguineous Pakistani

family with growth failure and features of relatively mild GHI[18]. In 2007, an additional seven 6Ψ patients were reported with a wider range of short stature (SS) phenotypes19. In 2013, Walenkamp et al described 2 further 6Ψ patients with growth failure followed by partial catch-up growth without treatment20. We recently reported the spectrum of clinical and biochemical features in 20 6Ψ subjects, which included eleven previously reported individuals and nine additional patients[21].

The *GHR* 6Ψ mutation (c.618+792A>G) is a homozygous point mutation in the final nucleotide of the pseudoexon, altering the 5' pseudoexon splice site in intron 6 of the *GHR* gene. This results in activation of the pseudoexon sequence, efficient splicing and inclusion of an additional 108 bases between exons 6 and 7. This results in the inclusion of 36 amino acids in the extracellular domain of the GHR protein[18] and functional work demonstrated that the mutant GHR protein impaired trafficking rather than signalling[22].

Under normal circumstances, the *GHR* pseudoexon is disregarded i.e. not spliced into the mature *GHR* mRNA. Previous work confirmed that binding of heterogenous nuclear ribonucleoprotein E1 (hnRNP E1) and U1 small nuclear ribonucleoprotein (snRNP) to the pre-spliceosomal complex prevented 6Ψ inclusion[8].

GHR 6Ψ mutation patients exhibit a wide spectrum of clinical and biochemical variability, even between individuals within the same kindred[21]. The height SDS of

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20 *GHR* 6Ψ patients previously described[21] varied between -1.7 and -5.9 with IGF1 SDS between -1.0 and -6.8. Additionally, 50% patients had 'classical' GHI facial features and the remainder had completely normal facial appearance[21].

Splice mutations may not always be 100% efficient in causing aberrant splicing and normal (wild type) and mutant transcripts may exist concurrently due to competitive use of normal and mutant splice sites. Previous data suggested that, alongside the abnormally spliced *GHR* transcript, a small amount of normally spliced wild-type *GHR* mRNA was present in *GHR* 6Ψ patients[18].

Analysis of cDNA of patients carrying different splice mutations of the same gene have shown that multiple abnormal splicing events occur alongside the production of the normal splice product, leading to a spectrum of phenotypes[23-25]. However, transcript variability has not previously been investigated in individuals with identical splice mutations.

The range of phenotypes observed in patients with the *GHR* 6Ψ mutation may be related to the presence of transcript heterogeneity i.e. the ratio of abnormal (mutant) to normal (wild type) *GHR* transcript. Genetic and environmental factors may also play a role in defining this ratio[19]. We investigated for the first time, whether *GHR* gene transcript heterogeneity and/or concurrent defects in other known short stature genes contributed to the observed clinical variability of 6Ψ subjects.

Subjects and Methods:

Subjects

The subjects were diagnosed with homozygous intronic *GHR* 6Ψ mutations at our centre between 2001 and 2014[18,19,21]. The referring physicians completed a proforma detailing the clinical and biochemical details at the time of DNA sampling for genetic analysis[21] and was prior to starting any growth promoting therapy. Height measurements were obtained using a wall-mounted stadiometer. Height was expressed as SDS according to the appropriate UK-WHO growth national standards[26,27]. IGF1 values were expressed as SDS based on the age and sex appropriate ranges provided by the host institution.

Fibroblast culture

Dermal fibroblasts from 4 *GHR* 6Ψ subjects (Patients 1-4) from 2 consanguineous Pakistani families were obtained by punch skin biopsies, which were performed according to established protocols[28,29] after written informed consent was obtained. Control human fibroblasts (normal neonatal male dermal fibroblast cell line) were obtained from American Type Culture Collection (ATCC, USA). Fibroblast cells were cultured in 75 cm^2 cell culture flasks (Greiner Bio-One, Germany) in High Glucose-DMEM (Sigma-Aldrich, UK) supplemented with 20% foetal bovine serum (Invitrogen), 50 units/ml penicillin and 50 µg/ml streptomycin.

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Reverse- transcriptase PCR (RT-PCR)

RNA was extracted from fibroblast cell lines using the RNAEasy kit (Qiagen, UK) as per the manufacturer's instructions. One microgram RNA was reverse transcribed using the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) mix (Thermofisher Scientific, UK) according to manufacturer's protocol. cDNA products from 4 patients and 1 control subject were amplified using the following intron skipping primers (**Figure 1a**):

1. Primers directed to exon 5 of the *GHR* gene (forward; AGTGCAACCAGATCCACC) and the junction of exons 6 and 7 (reverse; GGAAAATGATGGACCCTATA) to amplify the wild-type (WT-*GHR*) transcript (Reaction 1)
2. Primers directed to the *GHR* 6Ψ (forward; GGCACAGATCACTCCCAG) and the junction of exons 7 and 8 (reverse; GATTTCTACTTTCCATGGCTC) to amplify the mutant (6Ψ-*GHR*) transcript (Reaction 2)

Thermocycling conditions were: heated lid 110°C; 30 cycles at 95°C for 30s, 65°C to 55°C for 30s and 72°C for 30s followed by 10 cycles at 95°C for 30s, 60°C for 30s and 72°C for 30s. Products were visualised on a 2% agarose gel and verified by Sanger sequencing (GATC Biotech; https://www.gatc- biotech.com).

Quantitative RT-PCR (qRT-PCR)

The relative levels of WT-*GHR* and 6Ψ-*GHR* mRNA transcripts were quantified by quantitative RT-PCR using the primer sets above (Reactions 1 & 2). Expression

levels of the two transcripts were determined quantitatively by the MX3000 real-time PCR system (Stratagene) using SYBR green (Kapabiosystems, USA). 10 ng cDNA was added per 10 µL reaction. Thermocycling conditions were: 1 cycle at 95°C for 3 mins, 40 cycles at 95°C for 3s, 60°C for 20s and 72°C for 10s and 1 cycle at 95°C for 1 min, 60°C for 30s and 72°C for 30s.

The relative expression levels of the two transcripts were compared in the 4 patients and 1 control. *Gapdh* was used as the internal reference gene and 5 technical repeats for each patient were performed. For each experiment, five independent RNA extractions were assayed with three technical replicates. Relative mRNA expression (stated as mean±SD) was calculated by delta delta CT analysis (ΔΔCT) and values in patients and control were compared using one-way ANOVA with Bonferroni correction. $P<0.05$ was taken as statistical significance.

Genetic analysis by targeted gene sequencing

Genomic DNA was isolated from peripheral blood leukocytes (Qiagen DNeasy Kit) from 11 6Ψ subjects, including patients 1, 2 & 4 from whom skin fibroblasts were obtained. No DNA was available for patient 3. Targeted sequencing of the coding, promotor and intronic regions (2000 bp upstream and 500 bp downstream of each target gene) of 40 short stature genes was undertaken (**Table 1**). This was processed on an Illumina HiSeq 2500 sequencing platform with paired end of 100 and a designated average coverage of 100x (Otogenetics, Norcross, GA). The raw data from Otogenetics were analysed using DNA Nexus (DNAnexus Inc., Mountain View, CA, USA) by aligning to the *H. sapiens* GRCh37–b37 (1000 genomes Phase 1)

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reference genome with BWA-MEM FastQ Readmapper. VCF files were generated by Vendor Human Exome GATK-Lite Variant Caller (Unified Genotyper).

The resulting VCF files were uploaded to Ingenuity Variant Analysis (Qiagen, Germany) and results were analysed with the following filters: call quality ≥20, read depth ≥10, data outside 5% of most exonically variable 100 base windows in healthy public genomes. Common variants were filtered out by excluding those with a minor allele frequency of ≥0.5% in the 1000 genomes, ExAC and the NHLBI exomes.

Genetic variants were investigated *in silico* by SIFT (score 0, predicted deleterious to 1, predicted benign), PolyPhen-2 (score 0, predicted benign to 1, predicted deleterious) and CADD (Combined Annotation Dependent Depletion) score to predict the functional outcome. The CADD score assesses the negative effect of single nucleotide variants as well as insertion/deletions variants. A scaled CADD score of 20 represents a variant that is amongst the top 1% deleterious variants and a scaled CADD score of 30 means that the variant is in the top 0.1%.

Ethical approval

The study was approved by the Health Research Authority, East of England - Cambridge East Research Ethics Committee (REC reference: 17/EE/0178). Informed written consent for genetic research, skin biopsy and publication of clinical details was obtained from parents / carers and the patients where appropriate.

Results

Clinical phenotypes

The subjects studied had a range of clinical and biochemical heterogeneity as previously described. Patients 1 (height SDS -3.6 and IGF1 SDS -2.0) and 4 (height SDS -3.1 and IGF1 SDS -2.5) were first cousins from a consanguineous Pakistani family with no dysmorphic facial features. Patients 2 (height SDS -4.2 and IGF1 SDS -2.5) and 3 (height SDS -3.8 and IGF1 SDS -2.3) are siblings from another consanguineous Pakistani family. Both had typical facial features of GHI with mid-facial hypoplasia, depressed nasal bridge and prominent forehead. The mean age at presentation of the 4 patients was 3.2 years (range 2.6-3.8 years) (**Table 2**).

The mean age at presentation of the 11 6Ψ patients who underwent targeted gene sequencing was 4.8 years (range 1.2 to 9.9 years), mean height SDS -4.1 (range -3.0 to -5.1 SDS) and mean IGF1 SDS was -2.6 (range -4.0 to -2.0). 10/11 and 1/11 were from consanguineous Pakistani families and a non-consanguineous Indian family, respectively. Consistent with our previous report[21], 6/12 (50%) had facial features of GHI, as above.

Wild-type and mutant *GHR* transcript expression

In order to amplify the WT-*GHR* transcript, a RT-PCR reaction was performed (Reaction 1), using primers directed to exon 5 and the junction of exons 6 and 7 of the *GHR* gene (**Figure 1a**). The WT-*GHR* transcript (193 base pairs, bp) was identified in all 4 6Ψ subjects and the control. The 6Ψ-*GHR* transcript was

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amplified by a RT-PCR reaction (Reaction 2) using primers directed to the *GHR* 6Ψ and the junction of exons 7 and 8 of the *GHR* gene (**Figure 1a**). The mutant 6Ψ-*GHR* transcript (228 bp) was identified in all 6Ψ subjects but not the control (**Figure 1b**). Sanger sequencing verified all the predicted cDNA sequences.

Quantification of the wild-type and mutant *GHR* transcripts

qRT-PCR quantified the relative levels of the WT-*GHR* and mutant 6Ψ-*GHR* transcripts, using the primer sets above (Reactions 1 & 2).

WT-*GHR* mRNA expression (mean±SD) relative to control was 0.055±0.021, 0.022±0.014, 0.055±0.018 and 0.049±0.034 for patients 1-4, respectively (**Figure 2a**). This was significantly lower in all patients compared to control (1.001 ± 0.016); all p values <0.001. This suggests that only small amounts of WT-*GHR* transcript are present in the *GHR* 6Ψ patients.

Mutant 6Ψ-*GHR* mRNA expression was calculated relative to patient 1, rather than control, as mutant transcript expression was negligible in the control compared to the 6Ψ subjects. Mutant 6Ψ-*GHR* mRNA expression (mean±SD) relative to patient 1 (1.003±0.004) were 0.552±0.061, 1.003±0.180 and 0.40±0.069 for patients 2-4, respectively and 0.001±0.0003 for control. There was no significant difference in 6Ψ-*GHR* transcript levels between patients 1 & 3. However, 6Ψ-*GHR* transcript levels were significantly different between all the other patients (1 & 2, 1 & 4, 2 & 3 and 3 & 4; p<0.001 and patients 2 & 4;

p=0.017) (**Figure 2b**). This confirms variable amounts of mutant 6Ψ-*GHR* transcript in the *GHR* 6Ψ subjects, with negligible levels in control.

The mean 6Ψ:WT transcript ratios for patients 1-4 were 39:1, 71:1, 47:1 and 29:1, respectively (**Table 2**). These values correlated negatively with height SDS (R=-0.85, p value <0.001) (**Figure 2c**). This would suggest that shorter patients have transcript ratios in favour of the mutant *GHR*.

Genetic analysis

We analysed 11 6Ψ subjects (which included patients 1, 2 & 4) for genetic variants in 40 known human short stature genes. This revealed 8 predicted deleterious variants in 6 genes (*IGFALS, OBSL1, CBL, IGF1R, ACAN* and *CUL7*) in 8 of the 11 6Ψ subjects (**Table 3**). Patients 9 and 10 had compound heterozygous missense variants in *CUL7* and *IGFALS*, respectively (**Table 3**). The remaining 6/8 variants were monoallelic (patients 2, 4, and 5-8). 4/6 were missense and 2/6 were in-frame insertions. 6/8 variants had a CADD score of >20. Patients 4 and 6 had in-frame insertions in *CBL* and *IGF1R* genes (CADD score 12.6 and not known, respectively). None of the patients had homozygous variants identified.

We classified the pathogenicity of the variants according to the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP)'s standards and guidelines for the interpretation of sequence variants30 (Table 3). 7/8 of the predicted deleterious variants were variants of 'Uncertain Significance'30. Although the missense variants are very rare in

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populations (allele frequency <0.001 in the gnomAD database31) and in silico variant prediction tools support a deleterious effect on the gene, they fall into the category of 'uncertain significance' as they do not meet the criteria for being either pathogenic or benign30. Furthermore, although the IGF1R heterozygous c.109_123dupATCGACATCCGCAAC, p.I37_N41dup in-frame insertion variant fulfils 2 moderate criteria for pathogenicity30 i.e. protein length changes as a result of in-frame deletion in a non-repeat region and is absent from controls as well as large databases (allele frequency 0.002 in the gnomAD database31), it does not strictly meet the ACMG/AMP criteria for pathogenicity and hence is classified as 'uncertain significance'30. The CBL heterozygous c.125_127dupACC, p.H42dup variant is 'likely benign'30 as it is an in-frame insertion in a repetitive region without known function and there is 1 homozygote in the gnomAD database31.

Patients 2 and 8 with heterozygous ACAN variants did not have advanced bone maturation, early onset osteo-arthritis or intervertebral disc disease. Patient 6 (heterozygous in-frame IGF1R insertion) had normal birth weight, normal IGF-1 SDS, no developmental delay and consequently also did not fulfil the published criteria for the identification of patients with suspected IGF1R mutations (total score <3)32. Patients 4 and 7 with CBL variants had no cardiac defects or developmental delay. Therefore, although these defects could potentially contribute to the short stature, no phenotypic features associated with these heterozygous genetic defects were detected.

In the four patients who underwent transcript heterogeneity analysis, 3 underwent genetic sequencing of short stature genes. Patient 2 had a heterozygous missense *ACAN* variant. Patient 4 had a heterozygous missense *OBSL1* variant and a heterozygous in-frame *CBL* variant. Patient 1 had no predicted deleterious variants.

Reviewing all patients assessed on the gene panel (Table 3; n=11), there was no correlation between the number of predicted deleterious genetic variants (0, 1 or 2) and the degree of short stature (height SDS) in the individual 6Ψ patients, i.e. patient 4 had 2 variants and was not significantly shorter than those with 1 (n=7) or no variant(s) (n=3) (Table 3). Additionally, the mean (±SD) height SDS of patients with heterozygous or compound heterozygous variants in autosomal dominant inherited genes (*ACAN, CBL, IGF1R*) (-3.78±0.67) was not significantly different from that of patients with heterozygous or compound heterozygous variants in autosomal recessive inherited genes (*OBSL, CUL7, IGFALS*) or patients with no deleterious variants (-4.1±0.56), p=0.39.

Discussion

GH insensitivity (GHI) is a rare disorder caused by mutations in multiple GH-IGF1 axis genes. Homozygous *GHR* mutations are the commonest cause of 'classical' GHI and are associated with a wide range of clinical and biochemical phenotypes[33]. More recently, it has been noted that several other short stature

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disorders such as 3M, Noonan Syndrome (NS) and Silver-Russell Syndrome (SRS) overlap with GHI[2,34-37].

Milder or 'non-classical' GHI cases are being increasingly recognised owing to advent of next generation sequencing techniques and an increased awareness of this group of disorders. Other molecular defects of the GH-IGF1 axis that cause 'non-classical' or milder GHI phenotypes include dominant negative *GHR*[38-41] and STAT5B42 gene mutations, heterozygous IGF143-45, IGF246,47 and IGFALS48,49 mutations, homozygous PAPPA26 mutations and the GHR pseudoexon (6Ψ) mutation18-21. Non-classical GHI is an important clinical entity and the prevalence may be higher than classical GHI2. Approximately 70 different GHR gene mutations have been reported in more than 300 patients1,2. A recent UK study37 reporting the genetic diagnoses obtained from candidate gene and whole exome sequencing (WES) in a selected group of 107 patients with GHI showed that the GHR 6Ψ mutation contributed 25% (8/32) GHR mutations and 16% (8/51) of all genetic diagnoses.

The GHR 6Ψ mutation is notable as striking phenotypic variability is observed between different patients harbouring the same homozygous point mutation, even amongst members of the same kindred21. We hypothesise that several factors alone or in combination could contribute to the phenotypic variability observed between individual patients and include: 1. Differences in the levels of mutant vs wild type GHR transcripts 2. Concurrent genetic variants contributing to the degree of growth failure 3. Environmental factors and 4. Genetic variability in

the cellular processes that regulate the mutant and WT GHR proteins. These are discussed in detail below.

David et al19 suggested that the spectrum of clinical phenotypes observed in the GHR 6Ψ patients may be due to competitive use of both wildtype and 6Ψ mutant splice sites resulting in different ratios of the two transcripts. The current study tested the hypothesis that transcript heterogeneity could account for the phenotypic differences, particularly the severity of growth failure in the 6Ψ subjects.

We found variable levels of mutant 6Ψ-GHR mRNA expression among all patients except patients 1 and 3, who had similar height deficits of -3.6 and -3.8, respectively. Our work implies that the splicing of the GHR pseudoexon is highly inconsistent as variable quantities of mutant transcripts are produced in different individuals with the same mutation. Furthermore, consistent with our previous work18, we confirmed the existence of WT-GHR transcript in the 6Ψ subjects alongside the mutated 6Ψ-GHR transcript. This demonstrates that normal as well as abnormal splicing events co-exist in these subjects. Our results also confirm that a higher WT to mutant transcript ratio correlates negatively with the height SDS of the patients i.e. greater mutant pseudoexon inclusion may lead to a more severe phenotype.

Interestingly, consistent with previous published work18, no GHR 6Ψ transcript was detected in the control subject. This would indicate that under normal

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circumstances, the GHR pseudoexon splice site is not recognised by the splicing machinery thereby preventing its inclusion in the mature mRNA. This corroborates the work by Akker et al8 who showed that the heterogenous nuclear ribonucleoprotein E1 (hnRNP E1) and small nuclear ribonucleoprotein U1 (snRNP U1) bind to the relatively weak "wild-type" 5' GHR pseudoexon splice site in the pre-spliceosomal complex and silence it. The A>G base change at the 5' splice site in 6Ψ patients does not create a new splice site but increases the base pair match of the pre-mRNA with the snRNP U1. Therefore, the spliceosome recognises the GHR pseudoexon sequence and it is included in the mature mRNA of patients carrying the mutation19.

Genotype-phenotype correlations secondary to variable splicing have been reported in 3 previous studies. Zhu23 and Lemahieu et al24 investigated the association of different splice mutations of the WASP (Wiskott-Aldrich syndrome protein) gene with the clinical phenotypes of patients with WAS and X-linked thrombocytopaenia. Another study by Gurvich *et al*[25] investigated the correlation between the clinical phenotypes of 2 patients and the splicing efficiency of 2 different intronic pseudoexon mutations in the *DMD* (Duchenne muscular dystrophy) gene. All 3 studies demonstrated that relative amounts of mutant to normal transcripts positively correlated with the severity of the phenotypes of affected individuals. However, these studies examined different splice site mutations of the individual genes. Our work is the first to report the phenotypic impact of transcript heterogeneity in patients with an identical homozygous splice mutation.

The potential underlying mechanism(s) leading to a spectrum of phenotypes in subjects with the same genetic mutation are unclear. Gurvich *et al*[25] proposed that the difference in splicing efficiency between the 2 different *DMD* gene mutations could be attributed to the strength of the donor splice sites created by the mutation and/or the strength of the cryptic acceptor splice sites in the wild-type sequence which are further activated by the point mutations. This explanation would not be applicable to our patients as the 6Ψ intronic point mutation leads to activation of the same 5' donor splice site in all the subjects.

David *et al*[19] also suggested that the genetic background of individual patients and/or environmental factors may play a role in determining the differential use of WT vs mutant splice sites and thus generation of variable quantities of normal vs mutated transcript. Variations in the core spliceosome machinery (comprising hnRNP and snRNP) and variations in genes encoding RNA processing/RNA-binding factors can influence alternative splicing[50-52]. It is possible that differences in these factors between 6Ψ patients regulate the differential use of the mutated and WT splice sites and thus determine the amount of mutant vs WT transcript generated.

It is feasible that the GHI phenotype, particularly the severity of the growth failure observed in the 6Ψ patients, is influenced by genetic variants in different genetic loci. We sought concurrent defects in genes known to cause short stature, GH

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insensitivity and overlapping phenotypes e.g. Noonan, Silver-Russell and 3M Syndrome in 11 6Ψ patients.

Analysis of 40 genes (including intronic and promoter regions) revealed a significant proportion (73%) of 6Ψ subjects had predicted deleterious heterozygous or compound heterozygous genetic variants. Of these, 3 variants in ACAN, IGF1R and CBL genes are recognised to have an autosomal dominant pattern of inheritance leading to short stature53-55. It is possible that these variants contributed to the growth failure in patients 2, 4, 6, 7 & 8. However, none of these patients had additional phenotypic features that would be consistent with the identified additional genetic variant. Furthermore, there was no difference in height SDS between the patients with heterozygous/compound heterozygous variants in genes with an autosomal dominant pattern of inheritance compared to patients with heterozygous/compound heterozygous variants in genes with an autosomal recessive inheritance or patients with no deleterious variants. This implies that the *ACAN*, *IGF1R* and *CBL* gene variants identified did not impact significantly on the patient's phenotypes. Unfortunately, we were unable to perform segregation studies as parental DNA was not available for any of the subjects. However, consistent with our findings, a previous study of 2 6Ψ patients also demonstrated that other genetic variants did not contribute to the severity of the phenotypes20.

Interestingly, given that the majority of patients have consanguineous family structures, no homozygous variants were detected. We were also unable to

identify a correlation between the number of predicted deleterious variants and the severity of short stature in the individual GHR 6Ψ patients studied. This indicates that concurrent defects in this subset of known short stature, GHI and overlapping disorders genes do not appear to contribute to the growth phenotype in these patients. We cannot, however, rule out an impact of other known short stature genes or currently undiscovered short stature genes. It is also possible that multiple genes with smaller effect size could account for some or all of the clinical variation.

Genotyping a region spanning 17.10 Mb around the GHR gene on chromosome 5 and analysis of the complex single polymorphic region in intron 9 of the GHR in 5 GHR 6Ψ families showed the same genotype for all affected members, suggesting the presence of a common ancestor19. Furthermore, 19/22 the known GHR pseudoexon patients are of Pakistani ethnicity18-21. This implies that the *GHR* 6Ψ patient cohort share a common genetic background. This is comparable to the p.E180 splice *GHR* mutation, found predominantly in individuals from Ecuador, Brazil and Chile where a shared genetic background flanking the splice mutation was identified[56,57]. First degree relatives who are heterozygous carriers of the p.E180 mutation are modestly shorter than non-carrier relatives[58]. This is in contrast to our cohort, where heterozygous carrier parents of 6Ψ patients had normal stature[19].

It is recognised that a multitude of environmental factors such as nutrition, socio-economic status and adverse environmental conditions affect childhood

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growth[59,60]. It is possible that any of these factors contribute to the phenotypic variability observed in our patient cohort. However, as there are marked differences between affected individuals within the same kindred, environmental factors are unlikely to be very significant.

It is feasible that differences in processing, trafficking and degradation of the mutant and WT GH receptor proteins account for the clinical differences[19]. Maamra *et al*[22] confirmed that the mutant GHR 6Ψ protein compromised normal cell surface trafficking but signalling was unimpaired. Therefore, variability in genes regulating these cellular processes might conceivably have a greater influence on the mutant 6Ψ GHR protein than the WT GHR. Further work exploring this hypothesis is required.

It is important to acknowledge that our study has several limitations. Firstly, GHR transcript ratios were studied in only 4 6Ψ patients. Furthermore, 1 of the 4 patients was not assessed on the gene panel. Our work would not identify variants in other known short stature genes not included in the panel or defects in currently undiscovered short stature genes. Additionally, the phenotypic spectrum of individual genetic defects is expected to broaden as more patients are reported. Finally, we did not explore the mechanisms underlying the observed variable splicing or genetic variability which might affect GHR protein processing, trafficking and degradation. Further work is required to address these.

In conclusion, our work shows that there is a variable ratio of mutated and WT GHR mRNA transcripts in GHR 6Ψ patients. The mutated GHR 6Ψ transcript is not normally spliced in unaffected subjects. Patients with more severe phenotypes i.e. lower height SDS, have a GHR transcript ratio in favour of the mutant GHR. The reason for the variable splicing in different patients with the same mutation is fascinating. The genetic background of individuals may influence this. However, our preliminary work does not suggest that variants in candidate SS genes contribute significantly to the variability of growth failure. This is the first indication that variable splicing and transcript heterogeneity can lead to a range of short stature phenotypes in subjects harbouring the same genetic mutation.

Declaration of interest: None declared

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Author Contributions

SC, SJR, TM, HLS contributed to patient recruitment, data collection and analysis. SC, EC and LAM performed the genetic analysis. SC performed the molecular work and statistical analyses with support from AVM and JW. SC wrote the manuscript with input from LAM, MOS and HLS.

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Figure titles and legends:

Figure 1. Reverse Transcriptase PCR (RT-PCR) of wild-type (WT) and mutant transcripts in the 6Ψ and control subjects

a) Schematic diagram of the *GHR* gene showing the position of the 6Ψ pseudoexon and intron skipping primers. 6Ψ, mutant pseudoexon transcript; WT, wild type *GHR* transcript b) 2% agarose gel showing products of RT-PCR Reaction 2: 6Ψ transcript (228 bp) in all 4 6Ψ patients (patients 1-4) but not in the control subject. Bp, base pairs.

Figure 2. Quantitative RT-PCR (qRT-PCR)

a) Box and Whisker Plot with jitter showing qRT-PCR of WT *GHR* mRNA fold change relative to Control. Box plots show the mean, upper and lower quartiles and range; IQR= interquartile range; p values calculated by one way ANOVA with Bonferroni correction. Pt, patient. *** P value <0.001. b) Box and Whisker Plot with jitter showing qRT-PCR of 6Ψ-*GHR* mRNA fold change relative to Pt 1. Box plots show the mean, upper and lower quartiles and range; IQR= interquartile range; p values calculated by one-way ANOVA with Bonferroni correction. Pt, patient; ***p value <0.001; **p value=0.017. c) Scatter plot showing the

correlation between the height SDS at presentation and the mean 6Ψ:WT transcript ratios in the four 6Ψ patients**.** Pt, patient; 6Ψ, pseudoexon; WT, wild type; R, Pearson correlation coefficient.

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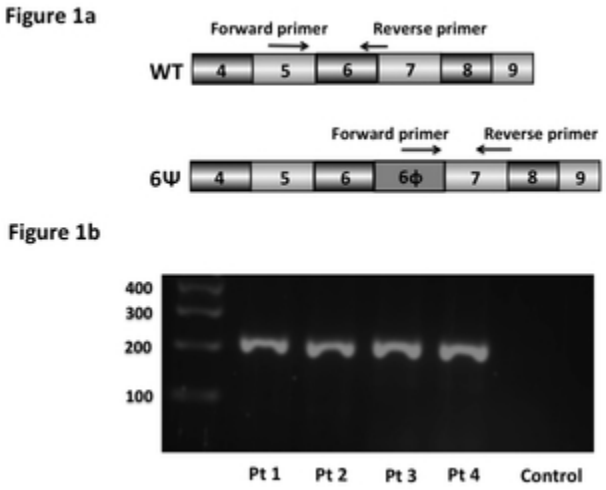

Figure 1. Reverse Transcriptase PCR (RT-PCR) of wild-type (WT) and mutant transcripts in the 6Ψ and control subjects

15x11mm (600 x 600 DPI)

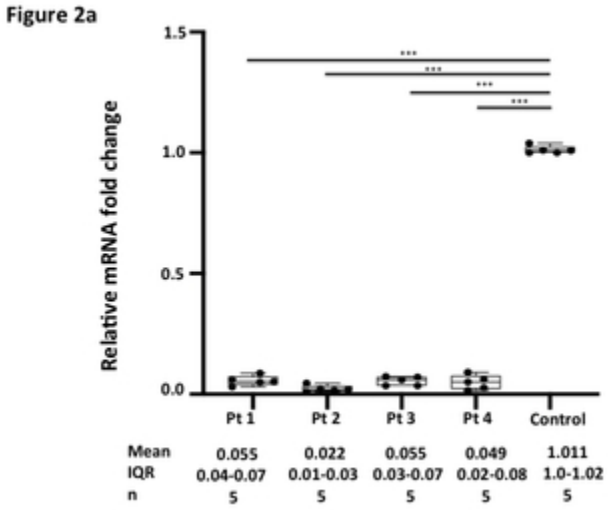

Figure 2a). Quantitative RT-PCR (qRT-PCR). Box and Whisker Plot with jitter showing qRT-PCR of WT GHR mRNA fold change relative to Control

15x11mm (600 x 600 DPI)

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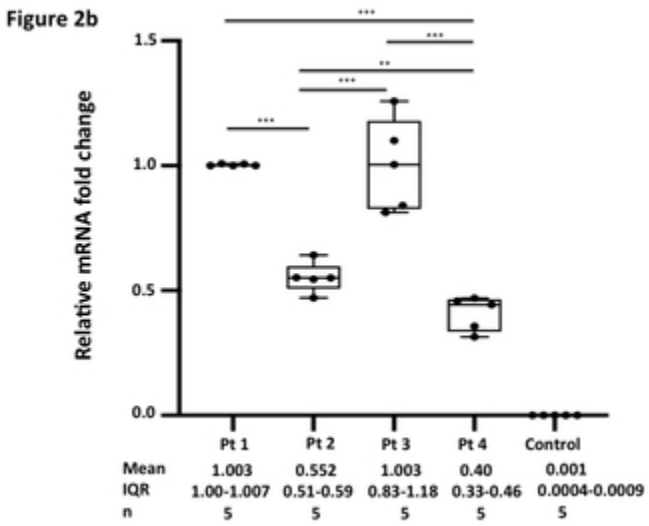

Figure 2b). Quantitative RT-PCR (qRT-PCR). Box and Whisker Plot with jitter showing qRT-PCR of 6Ψ-GHR mRNA fold change relative to Pt 1

15x11mm (600 x 600 DPI)

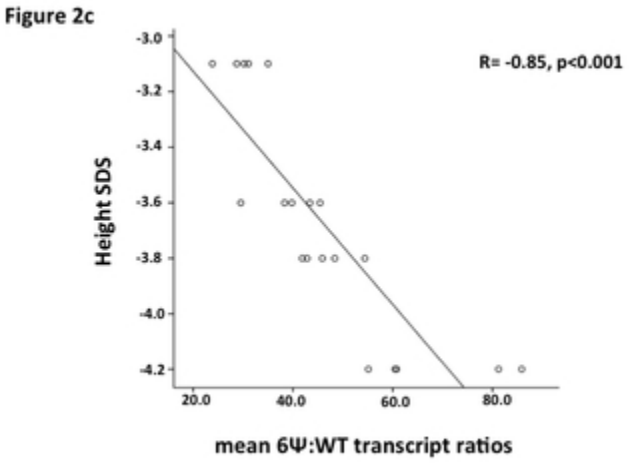

Figure 2c). Scatter plot showing the correlation between the height SDS at presentation and the mean 6Ψ:WT transcript ratios in the four 6Ψ patients.

15x11mm (600 x 600 DPI)

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Table 1. **Short stature genes included in the genetic analysis of 11 6Ψ subjects**

GHR	*IGFBP1*	*SOS2*	*MAP2K1 (MEK1)*
IGFALS	*IGFBP2*	*RAF1*	*MAP2K2 (MEK2)*
STAT5B	*IGFBP4*	*BRAF*	*A2ML1*
IGF1	*IGFBP5*	*NRAS*	*LZTR1*
PAPPA-2	*IGF2*	*KRAS*	*SHOC2*
IGF1R	*OBSL1*	*HRAS*	*ARAF*
IGFBP3	*CCDC8*	*RRAS*	*NF1*
PAPPA	*CUL7*	*CBL*	*NPR2*
STAT3	*PTPN11*	*RIT1*	*ACAN*
JAK2	*SOS1*	*RASA2*	*SHOX*

Genetic analysis included review of known (*GHR*, *IGFALS*, *STAT5B*, *IGF1, PAPPA2* and *IGF1R*) and putative (*IGFBP3, PAPPA, STAT3, JAK2, IGFBP1, IGFBP2, IGFB4, IGFBP5*) monogenic defects of the GH-IGF1 axis leading to GHI and IGF1 resistance phenotypes. We also sought variants in genes associated with overlapping short stature syndromes[2,34-37] 3M (*OBSL1, CCDC8, CUL7*), Silver-Russell (*IGF2*,) and Noonan (*PTPN11, SOS1, SOS2, RAF1, BRAF, NRAS, KRAS, HRAS, RRAS, CBL, RIT1, RASA2, MAP2K1, MAP2K2, A2ML1, LZTR1, SHOC2, ARAF, NF2*) syndromes. Other genes associated with short stature (*ACAN*[61,62]*, NPR2* [63], *SHOX*[64]) were also included in the analysis.

Table 2. Phenotypic features and 6Ψ-*GHR* to wild type-*GHR* transcript ratios in the four 6Ψ subjects

Patient	Sex	Age (yrs)	Height SDS	IGF1 SDS	Facial features	Mean 6Ψ/WT transcript ratio
1	M	3.8	-3.6	-2.0	N	39.2:1
2	F	3.7	-4.2	-2.5	Y	70.7:1
3	M	2.6	-3.8	-2.3	Y	46.9:1
4	M	2.8	-3.1	-2.5	N	29.4:1

M, Male; F, Female; Facial features of classical GHI (frontal bossing, mid-facial hypoplasia); N, No; Y, Yes; 6Ψ, mutant pseudoexon transcript; WT, wild type *GHR* transcript.

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Table 3. Genetic variants identified in known short stature genes in the 6Ψ subjects

Pt	Age (yrs)	Height SDS	IGF-1 SDS	Gene transcript	Translation	SIFT Score	Polyphen	CADD Score	ACMG/AMP classification
1	3.8	-3.6	-2.0	No deleterious variants detected	N/A	N/A	N/A	N/A	N/A
2	3.7	-4.2	-2.5	*ACAN* Het c.1223A>G, p.Q532H	Missense	Damaging	Prob Damaging	23.7	Uncertain significance
3	2.6	-3.8	-2.3	No DNA available	N/A	N/A	N/A	N/A	N/A
4	2.8	-3.1	-2.5	*OBSL1* Het c.2671A>G, p.T891A	Missense	Damaging	Prob Damaging	26.8	Uncertain significance
				CBL Het c.125_127dupACC, p.H42dup	In-frame	NK	NK	12.6	Likely Benign
5	5.7	-4.5	-2.5	*OBSL1* Het c.2671A>G, p.T891A	Missense	Damaging	Prob Damaging	26.8	Uncertain significance
6	1.2	-4.4		*IGF1R*	In-frame	NK	NK	NK	Uncertain

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			-2.2	Het c.109_123dupATCGACATCCGCAAC, p.I37_N41dup					significance
7	7.0	-4.2	-2.5	*CBL* Het c.2216C>T, p.S739F	Missense	Damaging	Benign	23.1	Uncertain significance
8	5.7	-3.0	-2.9	*ACAN* Het c.1596G>C, p.R132C	Missense	Damaging	Prob Damaging	27.9	Uncertain significance
9	4.3	-4.1	-4.0	*CUL7* Het c.464G>A, p.G155E c.620G>A, p.G207E	Missense	Damaging	Prob Damaging	26.9	Uncertain significance
10	2.5	-4.4	NK	*IGFALS* Het c.544C>A, p.L220M c.658C>A, p.L182M	Missense	Damaging	Prob Damaging	23.5	Uncertain significance
11	5.7	-4.7	-3.1	No deleterious variants detected	N/A	N/A	N/A	N/A	N/A
12	9.9	-5.1	-2.1	No deleterious variants detected	N/A	N/A	N/A	N/A	N/A

Pt, Patient; Ht, Height; NK, not known; Prob, probably; CADD, Combined Annotation Dependent Depletion; N/A, Not applicable; c. coding DNA sequence where nucleotide 1 is the A of the ATG-translation initiation codon (NCBI Reference Sequences: for *ACAN*, NM_013227.2; for *OBSL1*, NM_015311.2*;* for *CBL*, NM_005188.3; for *IGF1R*, NM_000875.3; for *CUL7*, NM_001168370.1 and for *IGFALS*, NM_004970.2); ACMG/AMP classification, classification as per the American College of Medical Genetics and Genomics and the Association for Molecular Pathology's standards and guidelines for the interpretation of sequence variants[30]. Patients 1-4 are the 6Ψ patients analysed for WT-*GHR* and 6Ψ-*GHR* transcript ratios (**Table 2**).