Characterization of an activating R1353H insulin-like growth factor 1 receptor variant in a male with extreme tall height

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Abstract

Objective: The insulin-like growth factor1 receptor (IGF1R) is important in growth and development, and inactivating *IGF1R* mutations cause short stature and relatively high levels of serum IGF-I. We identified an unclassified *IGF1R*^{R1353H} variant in a male with extreme tall height, very low levels of serum IGF-I and delayed and prolonged growth spurt. The index case's mother and three sons all carried the variant, but so far only the eldest son (age 18 years) presented with tall height. We hypothesized that the variant could constitute an activating mutation.

Design: The *IGF1R*^{R1353H} variant was investigated in *Igf1r*^{-/-} mouse embryonic fibroblasts (R-cells) by cell cycle, colony formation and transcriptome analyses.

Results: The *IGF1R*^{R1353H} (R-1353) exhibited significantly increased cell proliferation, G1-S progression and colony formation in soft agar. RNA sequencing identified 195 differentially expressed genes between R-WT and R-1353 (adjusted $P < 1E-100$). Most genes were upregulated in R-1353, including the gene encoding the androgen receptor (AR). Gene expression profiling showed the most significant enrichment in extracellular matrix organization ($P = 2.76E-7$), collagen biosynthesis ($P = 1.21E-5$) and cell adhesion ($P = 7.38E-5$). Retrospective biochemical analysis of the index case revealed decreased testosterone and sex hormone-binding globulin levels, whereas LH and FSH were within normal ranges. This profile suggests an increased sensitivity to androgen, which is compatible with the enhanced expression of *Ar* in R-1353 cells.

Conclusions: Our findings suggest that R1353H constitutes an activating *IGF1R* variant. The possible deregulation of collagen turnover and increased androgen sensitivity implicates an association to tall phenotype in male carriers.

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Introduction

The insulin-like growth factor-1 receptor (IGF-1R) is a cell-surface receptor tyrosine kinase (RTK) that is important for development, growth and progression of cancer (1). The IGF-1R is a central component of the growth hormone-IGF-I axis, which in turn is the most important system regulating mammalian longitudinal growth.

Heterozygous and compound heterozygous defects of *IGF1R* (or homozygous hypomorphic *IGF1R* mutations) cause partial IGF-I insensitivity and are associated with small birth size, short stature, small head circumference and relatively high circulating IGF-I levels (usually in the upper half of the reference range or above) (2, 3, 4, 5, 6, 7, 8).

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In patients with a heterozygous deletion spanning the *IGF1R* and surrounding genes (usually a 15q terminal deletion), additional clinical manifestations can be observed, including developmental delay, facial, cardiac and limb abnormalities. These abnormalities are believed to be caused by haploinsufficiency of the surrounding genes (2, 3, 4, 5, 6, 7, 8, 9). Duplication of the *IGF1R* caused by a trisomy of 15q26-qter is associated with tall stature, mental retardation and macrocephaly (10, 11). Cell proliferation studies on skin fibroblasts of a patient with three copies of *IGF1R* showed accelerated growth, increased IGF-1R phosphorylation and binding, while cells of a patient with only one copy of the *IGF1R* gene showed slower growth and decreased phosphorylation and binding (12). Thus, there seems to be a genotype/phenotype correlation between *IGF1R* copy number and stature.

It was long believed that RTK functionality is limited to cell membrane-bound ligand-activated signaling. Recent reports by our group and others have, however, shown a more diverse activity of RTKs, including nuclear translocation and activity of for example IGF-1R, FGFR and EGFR families (13, 14, 15). These findings may provide new understanding of the role of IGF-1R and other RTKs in normal physiology and disease.

In this study, we characterized the function of an *IGF1R*^{R1353H} variant of uncertain significance (VUS), which was identified in a male with extreme tall stature, a medical history of meningioma and low serum IGF-I levels. Given the clinical and biochemical presentation and the previously known role of *IGF1R* in statural growth, we hypothesized that this variant could help explain his phenotype. To test the *in vitro* effect of the R1353H variant, we compared *Igf1r*-knockout murine embryonic fibroblasts (R-) with expression of WT *IGF1R* (R-WT) and *IGF1R*^{R1353H} (R-1353). The results show that expression of *IGF1R*^{R1353H} increases cell cycle progression, cell proliferation and colony formation and associates with significant upregulation of genes involved in cell adhesion, collagen formation and extracellular matrix (ECM) composition, implicating that the *IGF1R*^{R1353H} variant results in a gain of function of IGF-1R.

Subjects and methods

Gene sequencing

Genomic DNA was isolated from peripheral blood samples using the Autopure LS Instrument (Gentra Systems, Minneapolis, MN, USA), and direct sequencing of *IGF1R*

(GenBank accession no. NM_000875.3) was carried out according to standard procedures at the Laboratory for Diagnostic Genome Analysis Leiden.

Reagents and cell lines

Polybrene, GAPDH antibody and normal mouse IgG were obtained from Santa Cruz. Anti-IGF-1R, Akt phosphorylation (pAkt), pErk, SUMO-1 and phosphotyrosine antibodies were purchased from Cell Signaling. BrdU, 7-AAD, mouse anti-IGF-1R and FITC-labeled anti-BrdU antibodies were purchased from BD Sciences. TaqMan real-time quantitative PCR (qPCR) primers, puromycin, protein G Dynabeads were provided by Life Technologies. The *Igf1r*-deficient R- cell line was isolated from mouse embryos with a targeted disruption of the *Igf1r* gene by Dr Renato Baserga's group. pBABE-Puro retroviral expression vector and Platium-E packaging cell line were bought from Cell Biolabs Inc. (San Diego, CA, USA).

Transfection of R-cell line

IGF1R^{R1353H} expression sequences were generated through introducing point mutation into WT *IGF1R* vectors previously generated in our group (16). Both sequences were sub-cloned into pBABE-puro vector and transfected into Platium-E cells for the production of retrovirus particles. The *Igf1r*-deficient R- cell line was seeded in 25 cm² flasks at 30% confluency and infected with 5 mL retrovirus supernatants with 8 µg/mL polybrene at 24, 48 and 72 h post seeding. pBABE-puro, pBABE-WT and pBABE-*IGF1R*^{R1353H} retrovirus particles were employed for mock, WT *IGF1R* and *IGF1R*^{R1353H} knock-in respectively. At 4 days after seeding, 2.5 µg/mL puromycin were supplemented in to the culture medium to eliminate non-transfected cells. Mediums were changed every third day until the flasks got 90% confluent. Limiting dilutions in 96-well plates were then carried out to isolate single clones from each transfection. Green fluorescent protein (GFP)-tagged WT IGF1R-coding sequence in pBABE-zeo vector was used for the generation of heterozygous cells. The procedure was the same as above except that 1200 µg/mL zeocin replaced puromycin and no cloning step was included.

Characterization of *IGF1R* expression levels

For chosen clones, the mRNAs were purified with RNeasy plus mini kits and reverse transcribed into cDNA with high-capacity RNA-to-cDNA Kit. TaqMan qPCRs targeting

IGF1R and *Gapdh* were carried out respectively, according to manufacturer's instruction. Relative *IGF1R* expressions were calculated through normalizing against *Gapdh* levels. Protein levels of IGF-1R were detected by western blot analyzing of total cell lysates. WT *IGF1R* and *IGF1R*^{R1353H} transfectants expressing equal *IGF1R* levels were selected and named R-WT and R-1353 (see 'Results' section).

Cell subcellular fractionation

Cells were cultured in 6cm dishes until 80% confluent. Proteins from subcellular compartments were extracted using the Qproteome Cell Compartment Kit, according to the manufacturer's instructions. Briefly, different buffers were sequentially added to pelleted cells to extract the cytosolic, plasma membrane and nuclear proteins respectively. The purity of each isolate was controlled by western blot detection of subcellular markers.

Immunoprecipitation and Western blot analysis

For each cell line, 10⁷ cells were boiled in 100μL TSD buffer (50mM Tris-Cl, 1% SDS, 5mM dithiothreitol (DTT), 20mM N-ethylmaleimide and 1× protease and phosphatase inhibitor) for 10min before sonicated briefly and centrifuged at 16 000g for 10min. The supernatants were diluted with 1.2mL of TNN buffer (50mM Tris-Cl, 250mM NaCl, 5mM EDTA, 0.5% NP-40, 20mM N-ethylmaleimide and 1× protease and phosphatase inhibitor). To pull down the IGF-1R, the lysates were incubated overnight with 5μL mouse anti-IGF-1R antibody and 10μL protein G Dynabeads at 4°C. Precipitated proteins were separated by SDS-PAGE, transferred onto a nitrocellulose membrane and blotted with specific antibodies. All IP/WB experiments were repeated for at least three times with uniform results. Representative pictures were chosen for presentation in the figure panels.

XTT cell proliferation assay

A total of 3000 R-puro, R-WT and R-1353 cells were each seeded in 96-well plates in complete medium respectively. The proliferation of cells was monitored every 24h with the Cell Proliferation Kit II (Roche). Five replicates were included in each time point.

Cell cycle analysis

R-puro, R-WT and R-1353 cells were seeded in 6cm dishes. When grown out to 70% confluence, the cells were starved

for 36h before stimulation with 50ng/mL IGF-I ligand. At 1h before harvest, 10μM BrdU was added to the culture medium. At specific time points (0–24h) post stimulation, the cells were harvested and fixed in 70% ethanol at –20°C overnight. Immunostaining was carried out with BD anti-BrdU antibody following the manufacturer's instruction. 7-AAD was employed to stain the DNA. The stained cells were analyzed with ACEA NovoCyte 3000 flow cytometry.

Soft agar assay

In six-well soft agar plates prepared as previous description (16), 1000 R-puro, R-WT and R-TSM cells were cultured at 37°C in a humidified incubator with 5% CO₂ for 2 weeks. The colonies in each well were stained with 200μL of nitroblue tetrazolium chloride solution overnight at 37°C for quantification. Five replicates were carried out for each cell line.

Whole transcriptome shotgun sequencing (RNA-seq)

Six separately seeded plates of R-WT and R-1353 were grown in basal conditions and harvested for total RNA using the Qiagen RNeasy plus mini kit. All samples showed good RNA integrity (>9.0) as determined by RNAscreenTape. Library preparation and sequencing were performed at the SciLifeLabs, Stockholm Sweden. Sequencing libraries were prepared using the Illumina Stranded mRNA (poly-A selection) kit and analyzed using paired-end 125bp sequencing at a HiSeq2500 system. The average sequencing depth was 35.8 million reads (min–max: 30.5–43.1M reads) with averagely uniquely mapped reads of 76.8% (min–max: 74.9–77.9%).

Bioinformatics and statistics

Reads were mapped to the mouse genome (NCBIM37, ensemble annotation 67) using Tophat v.2.0.4. Duplicates were removed using picard-tools v.1.29, and read counts were calculated with htseq v.0.6.1. Fragments per kilobase of exon per million fragments mapped was determined using cufflinks v.2.1.1. Differentially expressed (DE) genes were identified by analyzing the raw counts with the DESeq2 (v.1.10.1) package in R (v.3.4.0) and visualized in a heatmap generated by heatmap2, ggplot2 v.2.1.0 using non-supervised Euclidean clustering. Statistical overrepresentation (gene expression profiling) was analyzed using the Panther and Reactome databases including Bonferroni correction for multiple testing (19, 20, 21). A single gene was not annotated in either database

and was excluded from the gene expression profiling. A very low adjusted *P* value cutoff ($P < 1E-100$) was used, as explained in the 'Results' section.

Quantitative real-time PCR and statistics

To validate individual transcripts detected by RNA-seq, commercial probes were acquired (Mm00442688_m1 for androgen receptor (*Ar*) and Mm00438070_m1 for *Cyclin D2*). RNA was extracted from new cultures of R-WT and R-1353 cells and converted to cDNA as indicated earlier. The qPCR reactions were performed in an Applied Biosystems 7300 system and analyzed by SDS V1.4. All statistical comparisons were carried out using signal-factor ANOVA analyses, and *P* values < 0.05 were taken as statistically significant.

Results

The *IGF1R* R1353H variant presenting in a male with extreme tall stature

The index case was identified among a cohort of 18 very tall adults who consented to be investigated for genetic causes of extreme tall stature at the Department of Clinical Genetics, Leiden University Medical Center, Leiden, The Netherlands (the remaining cohort showed WT *IGF1R* sequence). He was investigated at 43.1 years, with a height of 209.5 cm (+3.6 SDS) (17) with a head circumference of +2.4 SDS and a BMI of 27.4. He had a birth length of +1.2 SDS (18), was tall as a child with delayed puberty (the growth spurt started at age 16 years) and continued growing until 20 years of age. At age 40 years, he was successfully operated for meningioma at the anterior border of the sphenoid. Neither symptoms nor signs of pituitary dysfunction have been noted, and serum FT4, TSH, cortisol and prolactin were all within the reference ranges. Yearly MRIs showed no anatomic abnormalities of the pituitary. As part of the investigation for tall stature, low serum IGF-I levels were observed (−3.0 SDS), after which *IGF1R* was sequenced. This revealed a heterozygous VUS in the intracellular domain of the receptor (rs149470389, g.99500625G>A, c.4058G>A, p.Arg1353His (R1353H)). Furthermore, a SNP array showed a normal male karyotype without alterations at the *SHOX* locus, and both the *NPR2* and *NPR3* genes were sequenced without showing any abnormalities.

The heights of his father and mother were +1.1 and −0.3 SDS respectively. He had three sons whose reported

heights at 2, 6 and 9 years of age were 90 cm (+0.5 SDS), 128 cm (+1.6 SDS) and 144 cm (+0.9 SDS) respectively, and at the age of 11.6, 15.6 and 18.4 years, reported heights were 160 cm (+1.0 SDS), 185 cm (+1.0 SDS) and 198 cm (+2.1 SDS) respectively. The height of the index patient's wife was 175 cm (0.7 SDS). He also had a sister who was operated for bilateral retinoblastoma. The mother and the three sons of the index case were found to carry the same *IGF1R* R1353H variant (Fig. 1).

Dermal fibroblasts from the patient were isolated and treated with different concentrations of IGF-I and compared with four dermal fibroblast cell lines (Co4, OB13, OB18 and OB20). Western blot measurement of pAkt was normalized against insulin receptor or tubulin. No significant difference in pAkt levels was observed between the patient's dermal fibroblasts and the control cell lines (Supplementary Fig. 1, see section on supplementary data given at the end of this article).

The *IGF1R* R1353H is a rare variant in the general population

The identified variant had a PhyloP score of 6.18 (−14.1;6.4). According to the genome aggregation database, the c.4058G>A variant is present in 71 of total 274440 alleles, of which 63 alleles were from a European (non-Finnish) population with 124760 alleles total, counting for a minor allele frequency (MAF) of 0.0005 (0.05%). The variant has also been reported in 2 of 8594 alleles (MAF=0.0002) in the European-American population of the Exome Sequencing Project, and in 2 of total 998 alleles (MAF=0.002) in the Genome of the Netherlands database, making it a rare variant. Since

Figure 1

Family pedigree of the index case with the identified *IGF1R* R1353H variant. The index case developed an extreme tall stature (+3.6 SDS) and low-serum IGF-I levels (−3.0 SDS). The variant was observed in the index case's three sons and mother. Variant carriers are marked by black shading. Squares indicate males, and circles indicate females. The arrow indicates the index case/proband (II:1). A full colour version of this figure is available at <https://doi.org/10.1530/EJE-18-0176>.

Figure 2

Characterization of R- cells stably transfected with WT and R1353H-mutated *IGF1R*. (A) Relative *IGF1R* transcription levels against *Gapdh* levels (Y axis) in R- clones were analyzed by qRT-PCR. Two cell lines with equal expression of WT-2C4 and R1353H-mutated (1353-2B1) *IGF1R* (both indicated with arrows) were selected for further investigation and named R-WT and R-1353 respectively. Another pair of cell lines, R-WT-2D5 and R-1353-3A2 (indicated by dotted arrows), with similar *IGF1R* expression levels were selected for validation experiments. No *IGF1R* expression was detected in R-puro where empty virus was used for mock transduction. (B) IGF-IR protein expression in R-puro, R-WT and R-1353 cells were assayed by western blotting using anti-IGF-IR β . GAPDH was blotted as loading control. (C) Western blots of IGF-1R in subcellular fractionations from R-puro, R-WT and R-1353 cells. The receptor was detected at equivalent levels in the membrane and nucleus fractions from R-WT and R-1353 cells and was undetectable in R-puro cells. N, K-ATPase and histone

the physicochemical difference between Arg and His is limited (Grantham distance: 29 (0–215)), we sought to characterize the functionality of this variant in an experimental setting.

IGF1R^{R1353H} increases cell proliferation and anchorage-independent growth

We used knockout (*Igf1r*^{-/-}) mouse embryonic fibroblasts cells (R-) stably transfected with WT *IGF1R* and *IGF1R*^{R1353H} as well as empty vector (R-puro). Based on the qPCR measurement of *IGF1R* mRNA expression levels, single clones with equivalent expression of WT *IGF1R* (clone WT-2C4, named R-WT) and R1353H-mutated *IGF1R* (clone 1353-2B1, named R-1353) were selected for further experiments (Fig. 2A). Western blot analyses also showed equivalent IGF-1R protein expression in both clones (Fig. 2B). As expected, R-puro showed no *IGF1R* gene or IGF-1R protein expression. Another pair of clones with equivalent *IGF1R* mRNA expression levels, WT-2D5 and 1353-3A2 (Fig. 2A), were selected for confirmation of biochemical results from R-WT and R-1353.

3 were blotted as fraction specific markers for membrane and nucleus proteins respectively. (D) R-puro, R-WT and R-1353 cells were serum-starved for 36 h followed with or without 10 min of IGF-I stimulation. Immunoprecipitated IGF-IR was used in western blot analysis with an anti-phospho-tyrosine antibody to investigate IGF-IR phosphorylation, re-blot with IGF-IR β was performed to confirm equal input. Separate western blot experiments were performed to investigate pAkt and Erk (pErk) in total cell lysates. GAPDH was used as loading control. (E) R-WT-2D5 and R-1353-3A2 were serum-starved for 36 h followed with or without 10 min of IGF-I stimulation. Immunoprecipitated IGF-IR was used in western blot analysis with an anti-phospho-tyrosine antibody to investigate IGF-IR phosphorylation, re-blot with IGF-IR β was performed to confirm equal input. (F) The kinetics of the IGF-1R WT and the R1353H variant were assessed by measuring the IGF-1R autophosphorylation using a phospho-IGF1R (Tyr1135/1136) antibody after IGF-I stimulation. R-WT and R-1353 were stimulated by 50 mg/ μ L IGF1 for 0, 5, 15, 30 and 45 min, and the total IGF1R β level was determined as loading control. All immunoprecipitation and immunoblotting experiments were successfully repeated at least three times. The figures show representative pictures from one of the experiments. A full colour version of this figure is available at <https://doi.org/10.1530/EJE-18-0176>.

R-puro, R-WT and T-1353 were also subjected to subcellular fractionation. The IGF-1R levels showed no difference in either the plasma membrane nor in the nucleus fraction between R-WT and R-1353 (Fig. 2C). The activity of IGF-1R signaling in the transfected cell lines was compared. Phosphorylation of IGF-1R, Akt and Erk was determined with or without stimulation with 50 ng/mL IGF-I of serum-starved cells. As shown, R-WT and R-1353 showed equal phosphorylation of IGF-1R, Akt and Erk (Fig. 2D). In R-WT-2D5 and R-1353-3A2, both WT and the R1353H-mutated IGF-1R also equally reacted to IGF-I stimulation (Fig. 2E). The kinetics of IGF-1R autophosphorylation were investigated after stimulation with 50 mg/ μ L IGF1 for 0, 5, 15, 30 and 45 min. No significant difference was found between R-WT and R-1353 (Fig. 2F). Cell proliferation of the different cell lines was compared. After 5-day growth in full-medium cell proliferation was determined by the XTT proliferation kit. R-puro increased 1.9-fold in cell number, whereas R-WT cells and R-1353 cells exhibited a 3.3-fold and 6.7-fold respectively (Fig. 3A) ($P < 0.05$ in days 2–5). Furthermore, colony formation in soft agar was investigated. After 2 weeks of culture in plates incubated with 0.3% soft agar, R-puro formed 1.6 colonies/well, R-WT formed 9.8/well and R-1353 formed 18.2/well ($P < 0.01$, compared to R-WT, Fig. 3B).

Cell cycle analysis of the cell lines after IGF-I stimulation for 0, 10, 16 and 24 h was determined by fluorescence-activated cell sorting (FACS) using BrdU/7-AAD staining (Fig. 4A). No significant cell cycle progression was observed in R-puro after IGF-I stimulation. Both R-WT

Figure 3

Proliferation and colony formation of R-puro, R-WT and R-1353 cells. (A) R-puro, R-WT and R-1353 cells were seeded in 96-well plates and cultured under basal condition. Cell proliferation was monitored with XTT proliferation assay kit every 24 h. The results are from five replications. (B) 1000 R-puro, R-WT or R-1353 cells were seeded in six-well plates with 1.0% soft agar and cultured for 2 weeks. The colony numbers in each well were determined using microscopy counting. The results are means from five replications.

Figure 4

Cell cycle progression of R-puro, R-WT and R-1353 cells after stimulation with 50 ng/mL IGF-I for 0, 10, 16 and 24 h. (A) Cell cycle progression (G1-S) was analyzed by FACS using BrdU/7-AAD staining. G1 (yellow), S (red) and G2 (purple) phases were gated respectively to investigate the cell cycle progression. (B) Percentage changes of S phase cells after 0, 10, 16 and 24 h of 50 ng/mL IGF-I stimulation are shown for R-puro, R-WT and R-1353 cells after normalization against unstimulated samples. Data represents three replications.

and R-1353 showed ligand-dependent increase in number of cells in the S phase at 16 h (Fig. 4A). R-WT exhibited a 23% increase, whereas the corresponding value for R-1353 was 35% (Fig. 4B). These data are in line with the cell proliferation results (Fig. 3), and together they provide strong evidence that the R1353H variant increases the pro-proliferative properties of IGF-IR.

IGF-IR^{R1353H} is associated with increased gene expression of extracellular and cell adhesion molecules

RNA sequencing was performed to compare gene expression of R-WT and R-1353. To achieve reliable data, six replicates of each cell line were analyzed. There were large differences between R-WT and R-1353 cells with

small differences between the biological replicates of each cell type, resulting in a large number of DE genes. Using a very low adjusted P value cutoff ($P < 1E-100$) DESeq2 identified 195 DE genes between R-WT and R-1353, the majority of which were highly expressed in R-1353 as illustrated by a heatmap (Fig. 5A). To identify co-expressed genes, the genes were clustered according to Euclidean distance into four groups (also shown in the heatmap Fig. 5A). Gene expression profiling were first performed for all DE genes ($n=195$) using Panther and Reactome pathways annotation databases. Significant enrichment was seen in the integrin signaling pathway ($P=8.02E-3$, 9 genes=5.45-fold enrichment), cell adhesion ($P=7.38E-5$, 18 genes=4.2-fold enrichment) and genes encoding ECM ($P=5.22E-6$, 12 genes, 7.63-fold enrichment) using the Panther database, as well as collagen biosynthesis ($P=1.21E-5$, 10 genes, 13-fold enrichment) and ECM organization ($P=2.76E-7$, 17 genes, 7.56-fold enrichment) using the Reactome database.

Gene expression profiling was similarly performed for each of the four groups of potentially co-expressed genes as determined by Euclidean clustering. Group 1 showed significant enrichment in the integrin signaling pathway, ECM proteoglycans and collagen formation. Group 2 showed significant enrichment of gene coupled to cell adhesion and ISG15 antiviral mechanism. Group 3 included a single gene and did not show any significant enrichment. Finally, Group 4 showed significant enrichment in genes coupled to ECM. All gene sets with corresponding fold enrichments and P values are shown in Table 1.

The IGF-IR^{R1353H} increases expression of the *Ar*

Among the 195 DE genes, we have validated RNA-seq data of individual genes whose functions could be coupled to cell proliferation and longitudinal growth. The expression of the *Ar* was determined by qRT-PCR in new cultures of R-WT and R-1353 cells, as well as in R-WT-2D5 and R-1353-3A2 cells, confirming significantly higher expression levels of *Ar* (74.0 ± 13.14 -fold and 55.6 ± 17.03 -fold respectively, Fig. 5B and C) in R1353H IGF-1R-expressing cells.

AR expression is also upregulated in WT/R1353H IGF-1R heterozygous cells

To investigate if cells with heterozygous expression of WT and R1353H *IGF1R* overexpress *Ar*, R-WT and R-1353 cells were transfected with GFP-tagged WT *IGF1R*. Zeocin

Figure 5

Gene expression differences between R-1353 and R-WT cells. (A) Heatmap of DE genes between R-1353 and R-WT. RNA sequencing was performed for six replicates of R-WT and R-1353. A total of 195 DE genes were identified by DESeq2 at adjusted $p < 1E-100$ and is depicted in the heatmap. Unsupervised Euclidean clustering separated genes into four expression clusters (Group 1–4). (B) Relative AR mRNA expression as determined by qRT-PCR in R-WT and R-1353 cells, showing 70-fold (74.0 ± 13.14) higher expression in the R-1353 cells. (C) Relative AR mRNA expression as determined by qRT-PCR in R-WT-2D5 and R-1353-3A2 cells, showing 55.6-fold (55.6 ± 17.03) higher expression in R-1353-3A2 cells. (D) Relative AR mRNA expression as determined by qRT-PCR in R-WT/WT and R-1353/WT cells, showing 51.1-fold (51.1 ± 7.14) higher expression in R-1353/WT cells. AR, androgen receptor; DE, differentially expressed.

treatment eliminated untransfected cells and the resulted cell lines were named R-WT/WT and R-1353/WT respectively. The successful expression of GFP-IGF1R was confirmed through microscopy observation where all cells had green fluorescence (Supplementary Fig. 2). qPCR revealed that AR was significantly upregulated in the heterozygous

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Table 1 Significantly enriched pathways and gene sets of differentially expressed genes from RNA-seq experiments.

	Number of genes (fold enrichment)	P Value
All differentially expressed genes (n = 194)		
Panther		
Integrin signaling pathway	9 genes (5.45)	8.02E-3
Cell adhesion	18 genes (4.2)	7.38E-5
Extracellular matrix	12 genes (7.63)	5.22E-6
Reactome pathways		
Collagen biosynthesis	10 genes (13)	1.21E-5
Extracellular matrix organization	17 genes (7.56)	2.76E-7
Group 1 (n = 64)		
Panther		
Integrin signaling pathway	6 genes (15.58)	3.04E-3
Reactome pathways		
ECM proteoglycans	5 genes (44)	1.68E-5
Collagen formation	6 genes (23)	3.65E-4
Assembly of collagen fibrils	5 genes (44)	9.9E-4
Integrin cell-surface interactions	5 genes (23)	4.36E-3
Group 2 (n = 107)		
Panther		
Cell adhesion	11 genes (4.77)	5.22E-3
Reactome pathways		
ISG15 antiviral mechanism	4 genes, 33 fold	1.05E-2
Group 3 (n = 1)		
No significant enrichment		
Group 4 (n = 22)		
Panther		
Extracellular matrix	3 genes (17)	3.87E-2
Reactome pathways		
No significant enrichment		

R-1353/WT as compared to the homozygous R-WT/WT (51.1 ± 7.14-fold, Fig. 5D).

Serum hormone levels of index case suggest increased androgen sensitivity

Given the increased levels of *Ar* in R-1353 cells, we hypothesized that the AR could be upregulated in the index case as well. Previously collected frozen serum samples were analyzed and revealed LH and FSH levels in the low normal range (3.4 and 2.8 U/L, reference ranges (RR): 2–9 U/L and 1.5–12.5 U/L respectively) and a decreased testosterone level (5.7 and 5.3 nmol/L at two different measurements, RR: 8–31 nmol/L). The level of serum sex hormone-binding globulin (SHBG) was also decreased (22.3 nmol/L, RR: 33.0–77.0 nmol/L), resulting in a calculated free testosterone of 117 pmol/L (RR: 120–750 pmol/L). Serum IGFBP-3 was 5.1 mg/L (RR: 2.6–6.1 mg/L).

Discussion

Inactivating *IGF1R* variants or a deletion of the *IGF1R* results in growth retardation, while gain of *IGF1R* copy

number due to a microduplication of 15q26.3 is believed to cause an overgrowth phenotype (10, 11, 12).

In this study, we identified and functionally assessed a rare and evolutionary conserved VUS of *IGF1R* found in a male with extreme tall height. Using transfected R-fibroblasts (*Igf1r*−/−), we showed that the *IGF1R* R1353H variant is associated with specific transcriptome changes as compared to the WT receptor. These include upregulating genes controlling cell adhesion and ECM composition, for example collagen formation. The gene expression differences were accompanied by increased cell proliferation, cell cycle progression and increased ability to form anchor-independent colonies.

Since, we hypothesized that the variant is coupled to the index case's height, we were initially puzzled by the fact that his mother has a normal height. Our finding that the R1353H variant strongly increases AR expression under both homozygous and heterozygous conditions (50- to 70-fold increases) opens the possibility of increased tissue androgen sensitivity. Measurements of circulating hormone levels of the index case revealed normal levels of LH and FSH, whereas the level of free testosterone and SHBG were low. Additionally, the low serum IGF-I cannot be explained by decreased IGFBP-3 as it was within normal

range. This hormone profile provides support for that the index case may have increased tissue androgen sensitivity *in vivo*. In turn, this could explain his tall height and why his mother lacks the tall phenotype. It is also in line with the observation that the index case's phenotype became pronounced during puberty. The three sons of the index case (all carriers) have shown some increased height, with the most prominent height in the eldest son, now 198 cm (+2.1 SD) at the age of 18 years. However, none has yet reached the age of the end of their father's growth period yet (approximately 20 years of age). If indeed the phenotype is restricted to male carriers, at least half of the carriers would be asymptomatic (<100/million people in the European-American population based on MAF).

The genotype–phenotype correlation observed in this family could also be explained by a potential parent-of-offspring effect. In patients with heterozygous deleterious *IGF1R* mutations or deletions, there is a larger proportion of maternally derived mutations in reported cases (11 maternal, 3 paternal) in cases discovered at the Leiden University Medical Center, in line with reported cases (J M Wit, personal communication). The described maternally differentially methylated region within intron 2 of the *IGF1R* could be involved in a potential parent-of-offspring effect (22), and its characterization would be very interesting in future investigations of *IGF1R*-related traits. However, biallelic expression of the *IGF1R* gene argues against a classic imprinting mechanism (23).

We also performed *IGF1R* gene sequencing in 130 patients with a phenotype resembling Sotos syndrome (but not carrying a mutation in *NSD1*) previously sent to our laboratory. A single patient, with a history of B-cell non-Hodgkin at 3 years of age, carried the *IGF1R* R1353H variant (maternally inherited) and presented with a very tall stature phenotype (+2.8 SD at 12 years of age) and fluctuating IGF-I levels (−0.36 SD at 8 years of age and 0.91 at 14 years of age). The maternal and paternal heights were −0.9 and −1.1 SDs respectively. The maternal grandmother did not carry the variant (paternal DNA not available). At 14 years of age, plasma testosterone was in the lower half of the reference range for early puberty (G2). The patient was later diagnosed elsewhere with Weaver syndrome (carrying a *de novo* constitutional *EZH2* mutation, p.Ala682Thr). Because of an extreme adult height prediction (204 cm, +2.84 SDS), epiphysiodesis at both knees was performed, which limited adult height to 199 cm (+2.14 SDS). It has previously been described that *EZH2* inhibits *IGF1* expression (24), underlining the complexity of interpreting the genotype–phenotype correlation in this patient.

The *IGF1R* R1353H variant has previously been described in a case of Ewing sarcoma and as a germ-line variant in a patient who presented with early onset of colorectal carcinoma (25, 26). No information regarding the height of these patients is available. As our *in vitro* studies showed that the R1353H variant significantly impact cell growth and anchorage-independent growth, the question may be raised whether it could increase the risk of cancer in carriers. The index case had a meningioma, but no malignancies for him or his children have been reported. Thus, the potential association of R1353H to tumor formation remains ambiguous.

The IGF-IR has been well characterized regarding its proliferative and anti-apoptotic effects through activation of the PI3K/Akt and MEK/Erk signaling pathways. In our experiments on transfected cells and patient-derived fibroblasts, the R1353H variant exhibited a kinase activity equivalent to that of the WT receptor as determined by IGF-IR, Akt and Erk phosphorylation after IGF-I stimulation. The kinetics of IGF-1R autophosphorylation implies that the *IGF1R* R1353H variant possesses comparable affinity for the IGF1 ligand. In agreement with this, primary cultures of the patient's fibroblasts showed similar IGF-I-induced Akt phosphorylation levels as compared to four dermal fibroblast cell lines. Studies by us and others have identified Akt- and Erk-independent functions of the receptor, for example histone modification or binding to transcription factors (27, 28, 29, 30). These functions are believed to be through the nuclear portion of IGF-1R (nIGF-1R). Even though nIGF-1R was discovered in cancer cell lines, recent evidence has shown the existence of nIGF1R in non-malignant cells, including normal diploid fibroblasts (31). Effects on such mechanisms could explain the observed gene expression differences. In transfected cells, the R1353H variant had no effect on the nuclear translocation; however, the variant might have interfered with the functions of nIGF1R, for example efficiency in binding to enhancer elements or co-factors. Furthermore, we have no data regarding the expression of the R1353H variant in the patient cells, which is a weakness of this study. Nevertheless, our data support the notion that IGF-IR functionality is not limited to Akt and Erk signaling.

In conclusion, this is the first characterization of an activating variant in *IGF1R* with implication to longitudinal growth. *In vitro* experiments showed that the *IGF1R* R1353H variant strongly increases *Ar* expression. Together with serum hormone profile of the index case, our findings suggest that this *IGF1R* mutant may lead to increased androgen sensitivity.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/EJE-18-0176>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this study.

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Author contribution statement

Conceptualization: Y L, H A D, J M W, O L. Methodology: Y L, C H, F H, O L. Formal analysis: Y L, H A D, F H. Investigation: Y L, H A D, H L, C Y, D W, S G K, F H. Resources: F H, J M W, O L. Writing – original draft: Y L, H A D, F H, J M W, O L. Writing – review and editing: Y L, H A D, H L, D W, C Y, S G K, F H, J W M, O L. Visualization: Y L, H A D, F H. Supervision: F H, J M W, O L.

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Clinical Study	Y Lin and others	Study of an activating IGF1R mutation	179:2	95
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