A novel variant of *FGFR3* causes proportionate short stature

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Abstract

Objective: Mutations of the fibroblast growth factor receptor 3 (*FGFR3*) cause various forms of short stature, of which the least severe phenotype is hypochondroplasia, mainly characterized by disproportionate short stature. Testing for an *FGFR3* mutation is currently not part of routine diagnostic testing in children with short stature without disproportion.

Design: A three-generation family A with dominantly transmitted proportionate short stature was studied by whole-exome sequencing to identify the causal gene mutation. Functional studies and protein modeling studies were performed to confirm the pathogenicity of the mutation found in *FGFR3*. We performed Sanger sequencing in a second family B with dominant proportionate short stature and identified a rare variant in *FGFR3*.

Methods: Exome sequencing and/or Sanger sequencing was performed, followed by functional studies using transfection of the mutant *FGFR3* into cultured cells; homology modeling was used to construct a three-dimensional model of the two *FGFR3* variants.

Results: A novel p.M528I mutation in *FGFR3* was detected in family A, which segregates with short stature and proved to be activating *in vitro*. In family B, a rare variant (p.F384L) was found in *FGFR3*, which did not segregate with short stature and showed normal functionality *in vitro* compared with WT.

Conclusions: Proportionate short stature can be caused by a mutation in *FGFR3*. Sequencing of this gene can be considered in patients with short stature, especially when there is an autosomal dominant pattern of inheritance. However, functional studies and segregation studies should be performed before concluding that a variant is pathogenic.

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Introduction

One of the most frequently mutated genes in patients with short stature is the fibroblast growth factor receptor 3 (*FGFR3*) gene. Mutations in *FGFR3* have been identified in several skeletal disorders (1), and almost all reported *FGFR3* mutations to date cause constitutive activation of the receptor, resulting in impaired endochondral bone growth (2).

Hypochondroplasia (HCH; OMIM 146000) is the least severe form of skeletal dysplasia caused by an *FGFR3* mutation. Although the expression of clinical features is variable, HCH is mostly characterized by short stature with disproportionately short limbs, relative macrocephaly, a normal face, and short and broad hands and feet. The most important radiologic features are shortening of long

bones, narrowing (or failure to widen) of the lumbar interpedicular distances, short femoral neck, and square ilia (3).

We studied two families in which several members had short stature, normal body proportions, and no evident radiologic skeletal abnormalities, transmitted in an autosomal-dominant fashion. In the first family, a novel missense mutation in *FGFR3* was revealed by whole-exome sequencing, which cosegregated with short stature and showed increased *FGFR3*, *STAT*, and *ERK/MAP* kinase activation *in vitro*. In the second family, Sanger sequencing revealed a rare variant in *FGFR3*, which did not fully cosegregate with short stature and did not activate *ERK/MAP* kinase.

Subjects and methods

Patients

Family A ► The index patient (Fig. 1; parents of the patient gave written permission for publication of these photographs) was a female of Dutch ancestry, born after an uneventful pregnancy at a gestational age of 41 weeks. Birth weight was 3025 g (−1.3 SDS) (4). Birth length was not measured. In the first years, her growth velocity decreased, but from 4 years onwards height remained stable at −3 SDS. No further health problems were noted.

Psychomotor development was normal. Her most recent examination has been at age 10.5 years, and shown a height (H) of 125.1 cm (−3.3 SDS) (5), weight 30 kg (BMI 19.2; 1.1 SDS) (6), head circumference (HC) 52.2 cm (−0.5 SDS) (7), span 129 cm, sitting height (SH) 66.8 cm (SH:H ratio 0.534, =1.0 SDS) (8). No physical anomalies were noted. A skeletal survey showed no abnormalities (Fig. 1). At a calendar age of 10.02 years, bone age was 9.89 years. Serum IGFI and IGFBP3 levels were within normal ranges. Karyotyping and *SHOX* mutation analysis showed normal results. SNP array (262K *NspI* Affymetrix array) revealed a 155.5 kb deletion of chromosome 9q31.3 (110.595.815–110.751.335; hg18), containing four genes, which did not segregate with the short stature in the family.

The pedigree is shown in Fig. 2. The mother also had short stature: H 151.4 cm (−3.1 SDS), HC 55.8 cm (+0.3 SDS), span 153.8 cm, SH 81.9 cm (SH:H ratio 0.541:1.1 SDS). Height of the maternal grandmother was 148.1 cm (−3.6 SDS), HC 54.2 cm (−0.7 SDS), span 150.7 cm, SH 80.4 cm (SH:H ratio 0.541, =1.1 SDS). The father had a normal stature and so did the brother of the index patient and the sister of the maternal grandmother.

Family B ► The index patient was a male of Dutch ancestry born after an uneventful pregnancy at a gestational age of 42 weeks. Birth weight was 3641 g (−0.3 SDS). Birth length was not measured. After 6

Figure 1

Clinical photograph (A) and X-rays (B, C, D and E) of the index patient at the age of 10.6 years. No evident radiologic abnormalities were noted (parents of the patient gave

permission for publication of these photographs). A full colour version of this figure is available at <http://dx.doi.org/10.1530/EJE-14-0945>.

Figure 2

Pedigrees of families A and B. Filled symbols represent family members with short stature. Underneath these symbols height SDS is displayed. M+, familial variant in *FGFR3* is present; M–, familial variant in *FGFR3* is not present. In both maternal grandfathers sequence analysis of *FGFR3* has not been performed. A full colour version of this figure is available at <http://dx.doi.org/10.1530/EJE-14-0945>.

months, his growth velocity decreased and from 4 years onward height remained stable at -3.2 SDS. No further health problems were noted. Psychomotor development was normal. His most recent examination has been at age 5.3 years, and shown H 99.7 cm (-3.1 SDS), weight 16.5 kg (BMI 16.3; 0.8 SDS), HC 50.5 cm (-0.6 SDS), and SH 57.0 cm (SH:H ratio 0.57, $=1.7$ SDS). No physical anomalies were noted. A skeletal survey showed no abnormalities. Bone age was 4.85 years at a calendar age of 4.23 years (within normal limits). Serum insulin-like growth factor 1 (IGF1) and IGFBP3 levels were within normal ranges. Whole-genome SNP array and mutation analysis for *NPR2* and *SHOX* showed normal results.

As shown in the pedigree (Fig. 2), the mother also had proportionate short stature: H 144.6 cm (-4.1 SDS), HC $+1.0$ SDS, span 149 cm, and SH:H ratio 0.54:1.7 SDS). The same holds for the 3-year-old sister (H 83.8 (-2.9 SDS), HC 49.5 (0.3 SDS), span 81.3 cm, and SH:H ratio 0.59 (0.8 SDS)). The sister of the mother and the maternal grandmother are both 146 cm (-3.8 SDS). The father was actually rather tall, 191.1 cm (1.0 SDS). The brother of the mother is 175 cm (-1.3 SDS).

DNA sequencing

The exomes of the index patient, her parents, brother, maternal grandmother, and the grandmother's sister in family A were sequenced and the variants were filtered

based on cosegregation, predicted impact on protein function, conservation and presence in public databases. The exomes were captured by the NimbleGen SeqCap EZ V2 kit followed by Illumina-paired end sequencing (2×100 bp) with at least $40 \times$ mean coverage. Downstream analysis was performed with an in-house pipeline (9). The variants discovered by whole exome sequencing were confirmed by Sanger sequencing. Sequencing of the coding region of *FGFR3* was performed according to standard procedures. Investigations were approved by the local ethical committee.

Western blotting, vectors and transfection

Rat chondrosarcoma (RCS) chondrocytes and 293T and NIH3T3 cells were propagated in DMEM medium supplemented with 10% FBS and antibiotics (Invitrogen). The cells were lysed in Laemmli buffer. Protein samples were resolved by SDS–PAGE, transferred onto a PVDF membrane, and visualized by chemiluminescence (Thermo Scientific, Rockford, IL, USA). Western blotting (WB) signal was quantified by Image J Software (National Institutes of Health, USA). The following antibodies were used: FGFR3, actin (Santa Cruz Biotechnology), ERK, pERK, pFGFR, pSTAT1, STAT1, pSTAT3, STAT3 (Cell Signaling, Beverly, MA, USA). The cells were transiently transfected using FuGENE6, according to the manufacturer's protocol (Roche). Vectors expressing V5-tagged human FGFR3 were made by cloning full-length human FGFR3 into the pcDNA3.1 backbone (Invitrogen); mutants were generated via site-directed mutagenesis (Agilent Technologies, Santa Clara, CA, USA).

Protein modeling

Homology modeling was employed to construct a three-dimensional structural model of the asymmetric dimer of the cytosolic tyrosine kinase domain (TKD) of the FGFR3. The crystal structure of the kinase domain dimer of the FGFR3 analog, namely epidermal growth factor receptor (EGFR) (PDB ID: 2GS6), was used as a template. The programs PHYRE (10) and UCSF Chimera (11) were used for generation of the dimer model.

Results

Whole-exome sequencing of all affected family members of family A revealed a novel mutation (NM_000142.4: c.1584G>T; Chr4(GRCh37): g.1807335G>T; p.Met 528Ile) in *FGFR3*. Detailed sequencing characteristics of

Figure 3

Schematic representation of location of the p.M528I mutation in the tyrosine protein kinase domain. A full colour version of this figure is available at <http://dx.doi.org/10.1530/EJE-14-0945>.

all performed exome sequencing analyses are listed in [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article. The p.M528I mutation is located in the TKD, close to the known HCH mutation p.N540K (Fig. 3). The variant was predicted to be possibly damaging in several *in silico* prediction programs (align GVGD: C0 (GV: 81.04 – GD: 10.12), SIFT: tolerated (score: 0.29, median: 3.24), MutationTaster: disease causing (*P* value: 1), PolyPhen: predicted to be probably damaging (HumDiv score 0.995 (sensitivity 0.68, specificity 0.97), and HumVar score 0.978 (sensitivity 0.58, specificity 0.94)). Sanger sequencing confirmed the presence of this mutation in the index, the affected mother and maternal grandmother, and was not present in the normal statured brother, father, and unaffected sister of the maternal grandmother.

Subsequent transfection studies of the mutant *FGFR3* into cultured cells proved the mutation to be activating, with the activation of *FGFR3*, *STAT*, and *ERK/MAP* kinase similar to the G380R mutation that causes achondroplasia (ACH) (Fig. 4).

Homology modeling showed a three-dimensional model of the asymmetric dimer of the cytosolic TKD of *FGFR3*, which highlights the position of Met528 at the dimerization interface essential for activation of the TKD (Fig. 5).

In the index patient of family B, a rare variant (NM_000142.4: c.1150T>C, r4(GRCh37): g.1806131T>C,

p.Phe384Leu) was found in the *FGFR3* gene with Sanger sequencing. This variant was also demonstrated in the affected sister and mother, but not in the affected grandmother and maternal aunt. The variant was predicted to be benign in several *in silico* prediction programs (align GVGD: C0 (GV: 204.97 – GD: 0.00), SIFT: tolerated (score: 1, median: 3.24), MutationTaster: polymorphism (*P* value: 0.918), PolyPhen: predicted to be benign (HumDiv score 0.003161–163) (sensitivity 0.98, specificity 0.44 and HumVar score 0.008 sensitivity 0.96, specificity 0.48)), which has been reported by Trujillo-Tiebas *et al.* (12) as a variant of unknown clinical significance and by Bodian *et al.* (13) as detected in a healthy ancestrally diverse cohort. The variant is described in dbSNP as rs17881656 (MAF/MinorAlleleCount: C=0.002/4) in populations from different ethnic origin. Transfection studies of the mutant *FGFR3* into RCS chondrocytes could not prove the mutation to be activating (data not shown).

Discussion

The association between a novel heterozygous p.M528I mutation in *FGFR3* and proportionate short stature shows that the already quite wide spectrum of clinical presentations of heterozygous *FGFR3* mutations should be further extended to proportionate short stature. This implies that for such patients *FGFR3* should be considered a candidate gene, particularly if short stature is

Figure 4

(A) RCS chondrocytes were transfected with WT FGFR3, FGFR3–M528I, known activating mutants (G380R, K650M), kinase-inactive mutant K508M, or empty plasmid. The cells were grown for 48 h and analyzed by western blotting (WB). The levels of ERK/MAP kinase activation by FGFR3 were determined by WB with antibody recognizing ERK only when phosphorylated (p) at T202/T204; pERK signal was quantified by densitometry and graphed (integrated optical density, IOD). Total ERK and actin levels serve as loading controls, total FGFR3 levels serve as control for transfection. Note the different levels of FGFR3-mediated ERK activation that are the highest in thanatophoric dysplasia mutation K650M. FGFR3–M528I activates ERK to the same extent as achondroplasia-mutant G380R. (B) 293T cells were transfected as indicated and analyzed for activating phosphorylation (p) of FGFR3^{Y653/Y654}, STAT1^{Y701}, and STAT3^{Y703} by WB. Signal was quantified by densitometry and graphed (IOD). Total FGFR3, STAT1, STAT3, and actin levels serve as loading controls. Note the increased activating phosphorylation of FGFR3, STAT1, and STAT3 (arrow) in two independent FGFR3–M528I transfections, compared with two wt FGFR3 transfections. (C) NIH3T3 cells were transfected as indicated and analyzed for ERK activation by WB (pERK). pERK signal was quantified by densitometry and graphed (IOD). Total ERK and actin levels serve as loading controls, total FGFR3 levels serve as control for transfection. Note the levels of ERK phosphorylation which are increased in FGFR3–G380R and FGFR3–M528I when compared with wt FGFR3. (D) Reproducibility of increased ERK activation by FGFR3–M528I in three RCS transfections compared with three independent transfections of wt FGFR3.

transmitted in an autosomal-dominant fashion. Our report also shows that functional studies are needed to confirm the pathogenicity of any novel or rare variant in this and other genes.

Recent functional studies for the ACH (OMIM 100800) mutations p.G380R and p.G375C have implied that these mutations increase the phosphorylation efficiency within unliganded FGFR3 dimers rather than increasing the cross-linking propensity of FGFR3 (14), although previous studies demonstrated that FGF ligand binding is essential for activation of mutated FGFR3 (15, 16). It is assumed that the FGFR3 mutations found in HCH also may result in constitutive activation of the receptor tyrosine kinase, but less than mutations causing ACH or thanatophoric dysplasia (3). The p.M528I mutation is located in the highly conserved intracellular TKD (conserved up to Tetraodon, see Fig. 3), just 12 amino acids away from the most common HCH mutation (p.N540K). The predictions according to software programs are similar to the predictions for the p.N540K mutation. We therefore anticipated the mutation p.M528I to be pathogenic and responsible for the observed phenotype. In order to functionally validate this prediction, we expressed FGFR3–M528I in RCS chondrocytes, which have successfully been used before to compare the relative levels of activation of different FGFR3 mutants associated with skeletal dysplasia (17). Figure 4A shows that FGFR3–M528I triggered ERK/MAP kinase activation in RCS cells to the levels similar to those induced by FGFR3–G380R, which is the most common mutation associated with ACH. Similarly increased activation of ERK, STAT1, or STAT3 was found in two other cell models transfected with FGFR3–M528I, e.g. 293T cells and NIH3T3 cells (Fig. 4A and B). In 293T cells, we also found an increased autophosphorylation of FGFR3–M528I when compared with wt FGFR3 (Fig. 4B). Collectively, these data confirm that M528I is indeed an activating mutation.

As mentioned earlier, M528 is situated in the cytosolic TKD. While the mutated site (M528) is outside all major active elements of the TKD, including the ATP binding site, activation loop, and/or substrate-binding site, our bioinformatics analysis suggests that it is located at the asymmetrical dimerization interface between two TKDs (Fig. 5). Formation of the asymmetrical TKD dimer appears essential for activation of the receptor tyrosine kinases from the auto-inhibited state (18). The activation of the TKD via asymmetrical dimerization presumes interaction of the C-lobe of the monomer B with the N-lobe of the TKD to be activated (monomer A) (Fig. 5) (18). Formation of the dimerization interface involving a conserved α -helix

Figure 5

(A) A general model of the dimerization-dependent activation of FGFR3. (B) Homology model of the asymmetric dimer of the cytosolic TKD of FGFR3 based on the crystal structure of the kinase domain dimer of EGFR (PDB ID: 2GS6) (10). The model highlights the dimerization interface (dotted line) essential for activation of the kinase. The activating mutation, M528I, is located in the conserved α C helix of the N-terminal lobe of the activated kinase. The α C helix is essential for formation of the dimerization interface and it participates in transduction of the allosteric signal from the dimerization interface to the kinase active site (10). (C) Local sequence alignment of the FGFR3 and the EGFR. Residues directly involved in formation of the dimerization interface as deduced from 2GS6 structure are displayed in the grey box. (D) Magnified view of the dimerization interface. The M528 adjoins a residue that is directly involved in the dimer formation. A full colour version of this figure is available at <http://dx.doi.org/10.1530/EJE-14-0945>.

(α C segment in Fig. 5) imposes allosteric modulation of the active site located between the N- and C-lobes of the TKD (18). Three alternative explanations for the activating phenotype of the M528I mutation can be deduced from the constructed model of the TKD dimer of the FGFR3 (Fig. 5). Firstly, the mutated residue might amplify allosteric signaling upon dimerization. Secondly, the mutated residue itself might be responsible for dissemination of the allosteric signal independent of the dimerization event. Thirdly, the M528I might promote TKD activation by stabilizing the interaction interface and promoting formation of the dimer. However, the latter explanation appears unlikely as the M528 residue is not directly involved in the dimerization interface, and the methionine and isoleucine are both hydrophobic amino acids with hydrophobicity values in the same range.

In addition to the most common p.N540K HCH mutation, other mutations have been reported to be associated with a milder phenotype with less severe disproportion, no macrocephaly, and minor radiologic abnormalities (19, 20, 21). Mild forms have been described, especially where specific mutations in the p.K650 codon are involved (22). In patients with these mutations, growth retardation is less severe and the lumbar interpedicular distances and fibula/tibia length ratios are closer to normal, although disproportion of the upper extremities is as severe as in patients with other HCH mutations. A mild phenotype has also been described in families with a p.N540S mutation (23, 24, 25). Patients with this mutation may have a height overlapping with the lower end of the normal range, but they all have radiologic abnormalities and some have a large HC.

The affected members of family A display an even milder phenotype, having short stature, a normal HC for age (though at the upper limit of the normal range if corrected for height SDS) (26), no disproportion, and a lack of radiologic signs of HCH. All affected family members had SH:H ratios around +1 SDS, which is to be expected for individuals with a height of approximately –3 SDS (8).

Family B shows an autosomal-dominant pattern of inheritance for short stature without disproportion and with a normal HC. Based on our finding in family A, we sequenced *FGFR3* and found the rare variant c.1150T>C (p.F384L) in the index patient, his sister and mother, who all shared the same growth pattern. However, further investigation in other family members (grandmother and maternal aunt) revealed that the mutation did not segregate with the short stature, which was in accordance with the prediction of a benign variant from several *in silico* prediction programs and the lack of altered ERK/MAP kinase activation in transfected RCS cells (data not shown). We therefore confirm that the *FGFR3* variant in family B is not pathogenic and another autosomal dominant cause of their short stature is suspected.

Our report contributes to the further delineation of the phenotype associated with *FGFR3* mutations. The reported mutation in family A is apparently linked to a phenotype of isolated short stature, whereas all other pathogenic mutations reported in *FGFR3* cause disproportion, relative macrocephaly, and/or radiologic abnormalities besides the growth retardation. We suggest considering sequence analysis of the *FGFR3* gene in patients with short stature, especially when there is an autosomal dominant pattern of inheritance, regardless of

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the body proportions. However, functional studies and proper segregation studies should be performed before concluding that the mutation involved is pathogenic.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EJE-14-0945>.

Declaration of interest

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

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