Novel and Recurrent Mutations in the *FGFR3* Gene and Double Heterozygosity Cases in a Cohort of Brazilian Patients with Skeletal Dysplasia

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Keywords

Achondroplasia · Double heterozygosity · *FGFR3* · High-resolution melting · Hypochondroplasia · Thanatophoric dysplasia

Abstract

Mutations in the fibroblast growth factor receptor 3 gene (*FGFR3*) cause achondroplasia (ACH), hypochondroplasia (HCH), and thanatophoric dysplasia types I and II (TDI/TDII). In this study, we performed a genetic study of 123 Brazilian patients with these phenotypes. Mutation hotspots of the *FGFR3* gene were PCR amplified and sequenced. All cases had recurrent mutations related to ACH, HCH, TDI or TDII, except for 2 patients. One of them had a classical TDI phenotype but a typical ACH mutation (c.1138G>A) in combination with a novel c.1130T>C mutation predicted as being pathogenic. The presence of the second c.1130T>C mutation like-

ly explained the more severe phenotype. Another atypical patient presented with a compound phenotype that resulted from a combination of ACH and X-linked spondyloepiphyseal dysplasia tarda (OMIM 313400). Next-generation sequencing of this patient's DNA showed double heterozygosity for a typical de novo ACH c.1138G>A mutation and a maternally inherited *TRAPPC2* c.6del mutation. All mutations were confirmed by Sanger sequencing. A pilot study using high-resolution melting (HRM) technique was also performed to confirm several mutations identified through sequencing. We concluded that for recurrent *FGFR3* mutations, HRM can be used as a faster, reliable, and less expensive genotyping test than Sanger sequencing.

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Activating mutations in the *FGFR3* gene located at 4p16.3 are responsible for some forms of skeletal dysplasia (SD). The *FGFR3* gene consists of 18 coding exons [Keegan et al., 1993; Le Merrer et al., 1994] and encodes an integral member of the family of fibroblast growth factor receptors (FGFR) that comprises 4 highly conserved proteins (FGFR1–FGFR4) [Liang et al., 2012].

During embryogenesis, the *FGFR3* gene is expressed at the bone growth plate where it regulates endochondral ossification, acting as a negative modulator of physiological growth of the skeleton [Xue et al., 2014]. Dominant mutations in this gene were first linked to achondroplasia (ACH; OMIM 100800), the most common form of disproportionate dwarfism in live newborns [Shiang et al., 1994; Unger et al., 2017]. Other phenotypes, such as hypochondroplasia (HCH; OMIM 146000) and thanatophoric dysplasia (TD; OMIM 187600) types I (TDI) and II (TDII) were later shown to be associated with specific mutations of that gene [Harada et al., 2009; Foldynova-Trantirkova et al., 2011]. Most of these autosomal dominant mutations frequently occur as de novo events. Their incidence has been shown to correlate positively with paternal age [Orioli et al., 1995; Barbosa-Buck et al., 2012]. Furthermore, Wilkin et al. [1998] demonstrated that the ACH mutation G380R occurs exclusively in the paternal allele.

SDs associated with *FGFR3* gene mutations exhibit a wide phenotypic spectrum; their severity is proportional to the extent of receptor hyperactivation and varies from mild forms, such as HCH, to more severe ones, such as the lethal forms TDI or TDII [Harada et al., 2009; Xue et al., 2014]. In ACH and HCH, short stature with disproportionately short arms and legs, genu varum, lumbar lordosis, and a large head are frequently observed. Due to phenotypic overlap, clinical diagnosis in some instances can be difficult to establish, making it important to test for the mutations underlying either disorder when ACH or HCH are suspected [Almeida et al., 2009; Xue et al., 2014]. Patients with TD exhibit lethal genetic abnormalities that lead to 2 types of manifestations: TDI with a very narrow thorax, very short limbs, femora curved as a “telephone hook,” and, occasionally, a “cloverleaf” skull. TDII is characterized by a similar phenotype, apart from straight femora and frequent presence of a cloverleaf skull [Foldynova-Trantirkova et al., 2011].

Herein, we report molecular screening of the *FGFR3* gene in a large cohort of Brazilian patients with ACH, HCH, and TD (types I and II) from 2 medical genetic centers in Brazil (the National Institute Fernandes Figueira/Fiocruz/Rio de Janeiro and the Medical Science Faculty

of the University of Campinas, São Paulo, Brazil). Additionally, a high-resolution melting (HRM) screening protocol was validated for the most common mutations associated with ACH, HCH, TDI, and TDII phenotypes.

Materials and Methods

One hundred and twenty-three patients with clinical and radiological characteristics of ACH, HCH, or TD (types I and II) were selected for molecular investigation.

Isolation of Genomic DNA

Peripheral blood samples were collected in EDTA-containing anticoagulant tubes from the patients admitted between 2012 and 2017 with clinical and radiographic diagnosis of SD likely related to *FGFR3* mutations. Genomic DNA was isolated from the blood by the standard phenol-chloroform extraction method. DNA from paraffin-embedded samples was extracted by an Illustra Nucleon HT kit (GE Healthcare).

DNA Amplification by PCR

Exons 7 and 17 (TDI), 10 (ACH), 13 (HCH), and 15 (TDII) known for recurrent mutations in the *FGFR3* gene were amplified in PCR with specific primers (primers are available upon request) using an Eppendorf Mastercycler Gradient thermal cycler (Eppendorf) and purified by an Illustra ExoProStar 1-Step kit (GE Healthcare) following the manufacturer's instruction. Primers were designed based on the *FGFR3* gene reference sequence (NG_012632.1).

Sequencing

PCR products were sequenced on an automated DNA sequencer ABI 3730 (Applied Biosystems) as described by Otto et al. [2008] using BigDye version 3.1 Sequencing Buffer (Applied Biosystems). Sequence data were analyzed using BioEdit Software version 7.2 (Ibis Biosciences).

Target resequencing was performed using a custom-designed sequencing panel for SDs on a NextSeq 500 platform (Illumina). Each mutation found was confirmed by Sanger sequencing.

High-Resolution Melting Analysis

Four mutations, c.1138C>A (ACH), c.1620C>A (HCH), c.742C>T (TDI), and c.1948A>G (TDII), previously detected by Sanger sequencing, were selected for the validation of the HRM technique as an alternative genotyping method. HRM pilot analysis was conducted using a 7500 Fast Real-Time PCR System (Life Technologies). The amplification was set up in a 20-μL volume containing 10 μL of Melt Doctor™ HRM Master Mix (containing AmpliTaq Gold® 360 DNA Polymerase, MeltDoctor*trade; HRM Dye, dNTPs, including dUTP, and MgCl₂), 10 pM of each primer, and 30 ng of isolated DNA. The amplification profile consisted of a single cycle of enzyme activation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s, and annealing/extension step at 60°C for 1 min. To determine the melting points, melting curve analysis was performed at 95°C for 10 s and 60°C for 1 min, followed by slow heating from 65°C to 95°C at a rate of 1°C/s. All reactions were tested in triplicate, using the wild-type allele to compare the melting curves. Data were analyzed using High-Resolution Melt Software (version 3.0.1; Life Technologies).

Fig. 1. Pedigree, X-ray plates, and clinical photography from case 1 with an atypical phenotype carrying *FGFR3* c.1138G>A (achondroplasia) and *TRAPPC2* c.6del (X-linked spondyloepiphyseal dysplasia tarda) mutations. The patient presented with an abnormal spine with platyspondyly and irregular vertebrae, especially in the dorsolumbar regions; hands showing a mild trident sign, normal finger length, and epiphyseal irregularities; feet also showing epiphyseal irregularities, and squaring of the iliac wings and iliac spiculae as those seen in achondroplasia. The lower limbs are

asymmetric, with a shortened, curved right femur, right tibia, and fibula. In addition, metaphyseal widening and epiphyseal irregularities were detected in the lower limbs. The upper limbs also show metaphyseal widening and epiphyseal irregularity with the right humerus and ulna slightly smaller and curved. A clinical photograph of the patient at the age of 35 months shows relative macrocephaly, protruding forehead, shortened and narrow thorax, mild trident sign, and an asymmetric right lower limb.

Table 1. Mutations identified in the cohort of 123 Brazilian patients with skeletal dysplasia

Clinical diagnosis	Molecular results	Protein alteration	Patients, <i>n</i> (%)	Patients, <i>n</i>	
				sporadic	familial
ACH SED-T-X	c.1138G>C (<i>FGFR3</i>)+ c.6del (<i>TRAPPC2</i>)	G380R+ Ser3Leufs*9	1/1 ^a	1 ^b	1 ^c
ACH	c.1138G>A g.1138G>C	G380R G380R	65/69 (94) 4/69 (6)	55/69 4/69	10/69 ^d 0/69
HCH	c.1620C>A c.1620C>G	N540K N540K	15/19 (79) 4/19 (21)	15/19 2/19	0/19 2/19
TDI	c.742C>T	R248C	16/28 (57)	16/28	0/28
	c.746C>G	S249C	5/28 (17)	5/28	0/28
	c.1118A>G	Y373C	4/28 (14)	4/28	0/28
	c.1130T>C+c.1138G>A ^e	L377P+G380R	1/28 (4)	NA	NA
	c.2419T>A	*807R	1/28 (4)	1/28	0/28
	c.2420G>C	*807S	1/28 (4)	1/28	0/28
TDII	c.1948A>G	K650E	6/6 (100)	6/6	0/6

ACH, achondroplasia; HCH, hypochondroplasia; NA, not available; SED-T-X, X-linked spondyloepiphyseal dysplasia tarda; TDI/TDII, thanatophoric dysplasia type I/II. ^a Case 1. ^b De novo mutation. ^c Maternally inherited mutation. ^d Cases 2 and 3. ^e Case 4.

Results

Among the 123 Brazilian patients with clinical and radiological diagnosis of ACH, HCH, TDI, or TDII se-

lected for molecular investigation, there were 69 cases of ACH, 19 cases of HCH, 28 cases of TDI, and 6 cases of TDII. Furthermore, one male 35-month-old patient, with an atypical SD phenotype (Fig. 1) was referred to

Fig. 2. Pedigree and X-ray plates from case 2 (achondroplasia) homozygous for the *FGFR3* c.1138G>A mutation showing severe platyspondyly, small iliac bones, marked shortness, and bowing of the long bones.

Fig. 3. Pedigree and X-ray plates from case 3 (achondroplasia) homozygous for the *FGFR3* c.1138G>A mutation demonstrating severe platyspondyly and short vertebrae, short thoracic cage, small iliac bones with spiculae, marked shortness, and bowing of long bones.

the hospital on suspicion of Morquio syndrome (properly excluded). His blood sample was investigated by the next-generation sequencing panel for SDs, and a combination of the classical ACH de novo c.1138G>A *FGFR3* mutation and a maternally inherited *TRAPPC2* c.6del (Ser3Leufs*9) mutation described as X-linked spondyloepiphyseal dysplasia tarda (SED-T-X; OMIM 313400) (Table 1) was revealed. Sanger sequencing confirmed both mutations. The patient's mother was tall and asymptomatic as was his unaffected father. The patient's phenotype was very peculiar. He was short (P10–P25) and exhibited macrocephaly (P75), with without frontal bossing. The disproportion of the segments was not evi-

dent (trunk and limbs). The thorax was small and narrow, the hands and fingers were not short, and there was a mild “trident” sign. Slightly bowed and asymmetric lower limbs were observed, with the lower right limb being significantly shorter than the left one. X-ray plates revealed an abnormal spine, especially in the dorsolumbar regions, severe metaphyseal abnormality, and minor epiphyseal dysplasia. The phenotype of the lower limbs suggested a typical ACH. The femur plate was abnormal with a very short neck and severe metaphyseal abnormality.

Homozygous c.1138G>A (p.G380R) genotype was found in 2 lethal ACH cases with mutations that were in-

Fig. 4. Babygram of 2 fetuses with thanatophoric dysplasia type I (TDI). Although the radiological findings were the same and characteristic of TDI in these 2 fetuses (the main findings being severe platyspondyly, small iliac bones with horizontal acetabular roofs and small sacroiliac notches, irregularity and flaring of metaphyses as well as marked shortness and bowing of long bones with femora shaped like a French telephone receiver), the mutations found in *FGFR3* were different. Whereas the first fetus (case 4) (**A, B**) has 2 heterozygous mutations, c.1130T>C and c.1138G>A, the other fetus has a typical c.742C>T TDI mutation (**C, D**) (see text).

herited from affected parents (cases 2 and 3) (Table 1; Fig. 2, 3). We also identified a third patient with a combination of 2 mutations in the *FGFR3* gene, c.1130T>C (p.L377P) and c.1138G>A (p.G380R), which displayed a typical TDI phenotype (case 4) (Table 1; Fig. 4). In this case, however, whether the mutations were in *cis* or *trans* configuration could not be verified.

Additionally, 2 TDI cases out of 27 and 1 ACH case out of 69 had homozygous genotypes for c.742C>T (p.R248C) and c.1138G>A (p.G380R), respectively, due to a polymorphism within the primer annealing region of the wild-type allele. Samples were sequenced with a new pair of primers and the heterozygous status of these patients was confirmed. Furthermore, 2 out of 69 ACH patients had false negative result due to a C>T substitution located at position g.16002 in the primer-annealing region on the mutated allele, already described in dbSNP. Clinical re-evaluation of both ACH patients supported the clinical diagnosis. New analysis using a different pair of primers correctly identified heterozygous c.1138G>A (p.G380R) mutations. In all cases, the amplification of one allele was prevented by the presence of polymorphism.

Finally, samples from 11 patients carrying recurrent mutations associated with each disease (ACH, HCH, TDI, or TDII) were submitted for the HRM analysis in order to optimize this fast genotyping method. The melting curves of the known mutations were compared to those in wild-type counterparts. Heterozygous variations c.1138G>A, c.1620C>A, c.742C>T, and c.1948>G could be differentiated from each other and from normal controls in all samples (Fig. 5).

Discussion

The results of molecular genetic testing for mutations present in a cohort of 123 Brazilian patients diagnosed with most common *FGFR3*-associated SDs were mostly in accordance with literature data. It is well known that ACH has low genetic heterogeneity [Bellus et al., 1995], and in approximately 97% of the patients, the c.1138G>A substitution, which leads to an exchange of glycine for arginine in the transmembrane domain of the *FGFR3* protein, is predominantly found. In this study, 65 patients (94%) presented with this mutation, confirming literature data. The remaining 4 patients (6%) presented with the c.1138G>C mutation, previously described in approximately 1% of the ACH patients [Bellus et al., 1995].

Among the 69 ACH patients, 2 unrelated cases that died in the neonatal period were homozygous for the c.1138G>A mutation. The mutations were inherited from both parents in these families (cases 2 and 3) (Fig. 2, 3). Indeed, the individuals with the homozygous autosomal dominant genotype usually display a severe phenotype, frequently leading to neonatal death [Foldynova-Trantirkova et al., 2011].

In our study, 15 out of 19 HCH cases (79%) carried the c.1620C>A mutation, which is the most common alteration related to this phenotype, occurring in approximately 50–70% of the cases. The c.1620C>G mutation was found in the remaining 4 patients (21%), occurring as a *de novo* event in 2 patients and as inherited familial mutation in another 2 patients.

Fig. 5. High-resolution melting analysis of the *FGFR3* gene. Melting curves in mutated and corresponding wild-type alleles are shown. Data from samples of patients are illustrated: **A** Achondroplasia (ACH). **B** Hypochondroplasia (HCH). **C** Thanatophoric dysplasia type I (TDI). **D** Thanatophoric dysplasia type II (TDII).

We also found 6 different genotypes in TDI patients: c.742C>T, c.746C>G, c.1118A>G, c.2419T>A, c.2420G>C, and c.1130T>C (the last mutation being concomitant with c.1138G>C). According to Xue et al. [2014], the c.742C>T and c.1118A>G mutations are the most frequent alterations found in TDI patients, contributing to about 90% of all cases. In contrast, in the present cohort, only 57% and 14% of patients presented with c.742C>T and c.1118A>G mutations, respectively, whereas 17% had the c.746C>G mutation. Collectively, these 3 mutations accounted for 88% of TDI cases in our study. Additionally, all patients with TDII presented exclusively with the c.1948A>G mutation known to be associated with this disease [Xue et al., 2014].

One patient with a TDI phenotype was found to carry 2 concomitant mutations: c.1130T>C (p.L377P) and c.1138G>A (p.G380R) (case 4; Fig. 4). The latter seems to be a rare and novel association. Rump et al. [2006] described almost the same alteration in a baby that presented with a severe ACH phenotype, and died at the age of

4 months. In that case, leucine in position 377 was replaced by arginine, whereas in the patient described in our study, leucine was substituted by proline. Both mutations were located at a highly conserved codon in the *FGFR3* gene that encodes of the transmembrane domain amino acids [Harada et al., 2009]. All prediction tools programs (PolyPhen-2, MutationTaster) pointed to p.L377P as a disease-causing mutation. This alteration may have an additive effect in *FGFR3* activation, similar to the effect of the homozygous p.G380R mutation, and most likely responsible for the severe phenotype observed in the patient.

Surprisingly, in the first evaluation of 2 TDI and 1 live ACH patients, 2 de novo homozygous mutations, c.742C>T and c.1138G>A, were found, respectively. These results were unexpected when considering that such mutations usually are autosomal dominant [Foldy-nova-Trantirkova et al., 2011]. Other 2 ACH patients were identified as being homozygous for the wild-type allele. However, such an atypical result, as was first noted

by Gusmão et al. [1996], was due to polymorphisms in the annealing region of the primer that prevented amplification of one allele. Indeed, heterozygosity for c.742C>T and c.1138G>A mutations was confirmed when a different pair of primers was used.

The double heterozygote case was investigated with a commercial next-generation sequencing panel for SDs. Two independent mutations related to ACH and SEDT-X were recognized; a de novo *FGFR3* c.1138G>A (Gly380Arg) mutation and a *TRAPPC2* c.6del (Ser3Leufs*9) deletion inherited from his normal mother.

Diagnosis of double heterozygosity describes cases when the affected individual has 2 different genetic disorders, each of which could be inherited from different parents, from the same parent, or one mutation could be inherited from a parent accompanied by a simultaneous de novo mutation in another gene [Flynn and Pauli, 2003]. Information regarding double heterozygosity has practical relevance for individuals with dominantly inherited bone growth disorders due to independent recurrent risks and variable combined phenotype. More importantly, the knowledge about the double heterozygosity phenotype is important with regards to potential severe health problems and survival of the infant. The seminal publication of Flynn and Pauli [2003] that reviewed literature data and reported 8 new observations, involving 4 double heterozygosity combinations, revealed that most frequently, ACH co-occurred with HCH, spondyloepiphyseal dysplasia congenita, pseudoachondroplasia, osteogenesis imperfecta type I/III, and Léri-Weill dyschondrosteosis. Clinical characteristics and natural history of individuals double heterozygous for the mutations leading to bone dysplasias are extremely varied and idiosyncratic to each combination [Flynn and Pauli, 2003]. In their study, the authors suggested that double heterozygous mutations causing bone growth disorders may be classified into 2 broad groups: those coding for proteins that interact directly with each other (anticipated to result in severe manifestations) and those that do not (resulting in additive or less than additive effects).

Despite the presence of the recurrent ACH c.1138G>A mutation in case 1, few clinical signs observed in ACH could be distinguished, including the typical pelvic and ischium shape, metaphyseal dysplasia, macrocephaly, and the trident signal of the fingers. Disproportionate body segments, kyphosis, frontal bossing and shortening of the limbs, usually observed in ACH patients, were not evident. However, an atypical body asymmetry was pres-

ent both clinically and radiologically. The right side was shorter than the left side, with curved bones, particularly in the forearm. From the radiological standpoint, an abnormal spine with platyspondyly and irregular vertebrae, especially in the dorsolumbar regions, could be observed. Regarding the spondyloepiphyseal abnormalities, epiphyseal irregularity along with metaphyseal widening in both limbs was prominent, which led to the SEDT-X clinical phenotype in the patient.

The increasing use of next-generation sequencing in clinical genetic diagnosis of SD cases will likely unveil double heterozygous genotypes more frequently.

In this study, samples from 11 patients with *FGFR3*-related SDs were analyzed using Sanger sequencing and HRM analysis. All cases carrying heterozygous mutations could be confirmed by both methods. Because SD cases often have recurrent mutations, HRM can be used as a genotyping test for the detection of recurrent variations using specific genotypes as control [He et al., 2012].

In summary, we studied a large cohort of 123 Brazilian patients with ACH, HCH, and TD (type I and II) and found 2 novel mutations: *FGFR3* c.1130T>C in a patient with a typical TDI phenotype and *TRAPPC2* c.6del related to SEDT-X in a patient with atypical ACH phenotype. Both mutations co-occurred with the ACH c.1138G>A variant. Other patients of this cohort presented with known recurrent mutations in the *FGFR3* gene. Furthermore, we demonstrated that the HRM method could be used as a faster, reliable, and less expensive genotyping test than Sanger sequencing for recurrent *FGFR3* mutations.

The molecular analysis is therefore important in SDs because it reinforces the genotype-phenotype correlation and establishes the molecular basis of atypical phenotypes.

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Statement of Ethics

Written informed consent was obtained from the parents prior to investigation. This study was approved by the Ethical Committee Boards of the National Institute Fernandes Figueira/Fiocruz and by the Medical Science Faculty of the University of Campinas (312.035 and 992/2007, respectively).

Disclosure Statement

The authors declare that they have no conflicts of interest.

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