Prenatal diagnosis of skeletal dysplasia due to *FGFR3* gene mutations: a 9-year experience

Prenatal diagnosis in *FGFR3* gene

M. J. Trujillo-Tiebas · M. Fenollar-Cortés ·
I. Lorda-Sánchez · J. Díaz-Recasens ·
A. Carrillo Redondo · C. Ramos-Corrales · C. Ayuso

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Abstract

Purpose Prenatal diagnosis with ultrasound findings compatible with skeletal dysplasia due to *FGFR3* mutations over a 9 year period in pregnancies and abortuses.

Methods 54 samples were studied. Aneuploidy studies were carried out on all samples. By sequencing analysis, we determined mutations for achondroplasia (ACH), hypochondroplasia (HCH), and type I and type II thanatophoric dysplasia (TD). **Results** 2 chorionic villi samples had a G380R mutation due to a mother with ACH; 4 amniotic fluid samples with TDs in which the foetuses had micromelia plus hypoplastic thoraces; 5 samples from abortuses with TDs. Neither ACH nor HCH occurred in sporadic cases.

Conclusions Molecular studies in ongoing pregnancies are indicated in cases with an affected parent, a family history

with positive molecular studies (maternal anxiety), and when the US finding demonstrates micromelia with a hypoplastic thorax. A protocol for tissues of abortuses should include an X-ray, pathologic anatomy, and genetic studies.

Keywords *FGFR3* gene · Prenatal diagnosis · Skeletal dysplasias · Ultrasound finding

Introduction

In 1988, Spranger classified hypochondroplasia (HCH), achondroplasia (ACH), and thanatophoric dysplasias (TD-I and TD-II) as the same family of dysplasias within the group of pathologies that are denominated generically as skeletal dysplasias [1]. All of the dysplasias display common phenotypic characteristics with different grades of severity.

The clinical observations of Spranger were subsequently confirmed. The fibroblast growth factor receptor 3 gene (*FGFR3*) is a transmembrane tyrosine kinase receptor that binds fibroblast growth factors (FGFs). The gene, isolated in 1991, is located on chromosome 4 (4p16.3) and is comprised of 19 exons and 18 introns [2, 3]. The codified protein consists of 840 amino acids and presents a highly conserved structure that is very similar to the rest of the proteins that compose the family of fibroblast growth factor receptors (FGFR-1, -2, and -4). Mutations associated with HCH, ACH, and TD are gain-of-function mutations causing ligand-independent activation of *FGFR3*. This generates a shortening of the long bones and abnormal differentiation of other bones [4]. All of the mutations are inherited in a dominant pattern.

TDs are the most frequent sporadic lethal skeletal dysplasias (OMIM:187600), with an incidence of 1–3:60000 [5, 6]. The physical characteristics of TDs include markedly

Capsule Molecular cytogenetic studies are indicated and complementary to ultrasound in familial cases involving mutations in the *FGFR3* gene.

Trujillo-Tiebas and Fenollar-Cortés contributed equally to this work.

M. J. Trujillo-Tiebas · I. Lorda-Sánchez · A. Carrillo Redondo ·
C. Ramos-Corrales · C. Ayuso
Department of Genetics, Fundación Jiménez Díaz (CIBERER),
Madrid, Spain

M. Fenollar-Cortés (✉)
Departamento de Genética, Servicio de Análisis Clínicos,
Hospital Clínicos San Carlos, C/ Prof Martín Lagos s/n,
Madrid 28040, Spain
e-mail: mfenollar.hcsc@salud.madrid.org

M. Fenollar-Cortés
Predoctoral fellow in Fundación Jiménez Díaz,
Madrid, Spain

J. Díaz-Recasens
Department of Gynecology, Fundación Jiménez Díaz,
Madrid, Spain

shortened limbs, macrocephaly, platyspondily, and a narrow thoracic cage with short ribs. Individuals usually die after birth from respiratory distress secondary to pulmonary hypoplasia. Two phenotypes have been distinguished: 1) the above-described clinical signs plus curved femurs (TD-I) or 2) the less frequent, straight femurs and a cloverleaf skull (TD-II). There are several mutations described in TD-I that result from either a stop or missense mutation in different codons (mainly codons 248, 650, and 807), but in TD-II an exclusive mutation (Lys650Glu) has been associated with this phenotype [4, 7, 8].

Achondroplasia (ACH; OMIM: 100800) is the most common type of human dwarfism, with an incidence between 1 in 10,000 and 1 in 30,000 live births [5, 6, 9]. ACH is characterized mainly by disproportionate short stature, macrocephaly, spinal stenosis, brachidactyly, and three-pronged fingers (trident). Greater than 90% of the cases are sporadic and are strongly associated with paternal age [4, 10, 11]. The first mutation described in the *FGFR3* gene was at codon 380 (G>A transition at nucleotide 1138). This transition is observed in 95% of affected patients [12–14]. In the same nucleotide, another change was identified as a G>C transversion [13]. Both mutations result in the substitution of an arginine amino acid for a glycine at codon 380 that is located in the transmembrane domain of the protein.

HCH is characterized by short stature, micromelia, and lumbar lordosis (OMIM: 14600). The phenotype is similar, but milder compared to ACH. Mutations are more widespread in *FGFR3* with a hotspot in the tyrosine kinase domain at codon 540 in exon 13, in which diverse mutations have been detected [15]. Codons 538 and 650 have been implicated in this pathology as well. However, only 70% of clinically diagnosed patients have a known mutation and there are reported familial cases not linked to chromosome 4 that supports a clinically and genetically heterogeneous condition [4, 16].

Accurate prenatal diagnosis of skeletal dysplasias based only on ultrasonographic (US) and radiographic results is feasible only for the lethal types [17] and most of them are detected in advanced pregnancy. The introduction of molecular analysis has played an important role for accurate diagnosis in the uterus.

The aim of the present study was to report on our experience over 9 years in *FGFR3* molecular prenatal diagnosis with suspicion of skeletal dysplasias. In addition, we wanted to define a protocol for best practice on gravidas with a US-positive finding and abortuses with phenotypes compatible with skeletal dysplasia.

Materials and methods

Fifty-four prenatal samples were referred to the Laboratory of Genetics for molecular analysis of the *FGFR3* gene with

the diagnosis of a skeletal dysplasia or a specific dysplasia (HCH/ACH) from different Spanish Hospitals over 9 years. Nineteen samples were from chorionic villi at 10–19 weeks gestation (CVS, 35%); 15 amniotic fluid samples at 13–35 weeks (AF, 28%), and 20 were samples from abortuses at 12–23 weeks gestation (TA, 37%). In addition, six parental samples were requested to identify the mutation in affected parents with an ACH or HCH phenotype. Samples had been provided to the Department of Genetics since 1999 by public and private clinics to confirm a skeletal dysplasia phenotype. The invasive technique after week 15, transabdominal chorionic villus biopsy or amniocentesis, was decided by the gynecologist depends on echographic parameters and amount of DNA test needs. Since 2002, our laboratory has been certificated by the European Molecular Quality Network (EMQN).

Of the 19 CVS samples, the indications were as follows: i) US finding of skeletal dysplasia (marked micromelia or lethal skeletal dysplasia) in an ongoing pregnancy in 5 samples between 15–20 weeks gestation; ii) previous fetal ACH diagnosis by US, but not tested by molecular studies in 7 samples; and iii) an affected familial member with positive molecular studies in 7 cases (4 maternal ACH, 2 offspring ACH, and 1 fetal TD-I).

From 15 AF samples, i) 13 were referred because of alteration during a routine US examination (short limbs were the most frequent finding) and 4 had a narrow chest and micromelia; and ii) 2 were referred because of a male sibling with ACH and a previous fetal ACH diagnosis by US, but not tested by molecular studies.

From 20 TA samples, indications were skeletal dysplasia in 16 cases, TD in 2 cases, and ACH in the remaining 2 cases.

Molecular studies

Genomic DNA was extracted from lymphocytes, TA, AF, and CVS with BioRobot EZ1 (QUIAGEN, Germany), with DNA-blood, DNA-tissue, or forensic cards and reagents of blood or tissue according to the sample (DNA Blood 350µl extraction Kit; Tissue Kit).

Chromosomal abnormalities were ruled out by cytogenetic or molecular studies in all cases. Two Multiplex QF-PCR assays with STR markers for chromosomes 13, 15, 18, and X were performed for the study of aneuploidies [18].

Sequencing analysis was chosen as the technique for molecular analysis of mutations because it generates more information and therefore allows a diagnosis based on a more complete molecular study. Analysis of all the mutations associated with the *FGFR3* gene is advisable, so we made five PCR reactions in the study routine, starting with the mutation which the phenotype suggested. Primer pairs used to amplify part of exons 6, 7, 10, 13–16, and 19 are available in Table 1 [7, 13, 15]. Five PCR reactions were performed in

Table 1 Sequences of primer pairs used to amplify exons 7, 10, 13–16, and 19

Primers	Length (pb)	Codon	Exon	Ta
5'GTCGTGGAGAACAAGTTTG3' 5'GCAAGGTGTACAGTGACGCA3'	158	TD-I: 248, 249	7	58°C
5'AGGAGCTGGTGGAGGCTG3' 5'CCACCGTGCACAAGATCTCC3'	224	TD-I: 370, 371, 373 ACH: 375, 380, 394	10	65°C
5'ACTGACAAGGACCTGTCGGACC3' 5'GACTACTCCTTCGACACCTGCAA3'	146	HCH: 538, 540	14	66°C
5'GACCGAGGACAACGTGATGAAGAT3' 5'CCTTGTTTGACCGAGTCTACACTC3'	143	TD-I, TD-II, HCH: 650	15	65°C
5'CCTGTCGGCGCCTTTTCGAGCAGTAC3' 5'CTAGCAGCCCACCCTGCTGCTGGTG3'	194	TD-I: 807	19	70°C

Ta: temperature annealing

a 50 µL reaction volume containing 10 µM of each primer, 200 µM dNTPs, 10X FastStart Taq DNA polymerase buffer (15 mmol/L MgCl₂; Applied Biosystems, USA), GC-RICH solution 5X (Roche), and 0.5 U/µL of FastStart Taq DNA polymerase (Applied Biosystems, USA). A 100 ng aliquot of genomic DNA was denatured for 10 min at 95°C followed by 35 cycles of amplification (30 sec at 95°C, 30 sec at different Annealing (Table 1), and 45 sec at 72°C), followed by a 10 min extension at 72°C. PCR products were directly sequenced using the Big Dye Sequencing kit (Applied Biosystems, USA) on an ABIPRISM 3130 Genetic Analyzer and analysed with the Sequencing 5.2 software package (Applied Biosystems).

Results

Eleven of the 54 samples were found to carry a mutation on the *FGFR3* gene (Table 2). Only 2 CVS samples were positive and both because of a maternal ACH. In the AF samples, just as in the four cases with a hypoplastic thorax plus micromelia were detected, there was a positive molecular test, as follows: 3 cases of TD-I (2 with a 742C>T [R248C] mutation and 1 with a 1118A>G [Y373C] mutation) and 1

case of TD-II (1948A>G [K650E] mutation). In another four AF samples with negative *FGFR3* results, another pathology was diagnosed later: i) polycystic kidneys, ii) short rib polydactyly syndrome, iii) a neonate with a cardiovascular malformation and cleft palate; and iv) a probable intrauterine growth retardation secondary to corticoid treatment in a mother with systemic lupus erythematosus [19].

Five mutations were identified in samples from abortuses: 2 TD-I and 3 TD-II. Figure 1 shows an X-ray of two of the samples with the most characteristic signs. Four samples were not able to be studied due to degraded DNA, but pathologic studies of one of the samples indicated a probable 18 trisomy. One sample from an abortus was a 69,XXY triploidy. A non-reported T-to-C change at 1150 nucleotide (F384L) of the *FGFR3* gene was found in other sample from an abortus. The father carried out the same change without any suggested clinical criteria [20]. We propose these polymorphisms/mutations as an additive pathologic effect in the receptor in combination with other changes in the same or other family member receptor of FGF, but further studies are necessary to confirm the hypothesis suggested.

Four of the 6 parental samples were carried out for the G380R ACH mutation. Two were necessary for checking the 384 polymorphic mutations.

Table 2 Mutations on the *FGFR3* gene

	CVS			AF			TA	
	USOP	PMS-	AFMS+	USOP	PMS-	AFMS+		TOTAL
	5	7	7	13	2	0	20	
TD-I	0	0	0	3	0	0	2	5
TD-II	0	0	0	1	0	0	3	4
ACH	0	0	2	0	0	0	0	2
	2/19 (11%)			4/15 (27%)			5/20 (25%)	11/54 (20%)

CVS: chorionic villi samples. AF: amniotic fluid samples. TA: tissue of abortion. USOP: US finding of skeletal dysplasia in an ongoing pregnancy. PMS-: previous fetal ACH diagnosis by US, but not tested by molecular studies. AFMS+: an affected familial member with positive molecular studies.

Fig. 1 Anteroposterior postmortem plain X-ray of two thanatophoric dysplasia affected fetuses: **a** Type I with hypoplastic thorax with shorts ribs, markedly shortened limbs with curved femurs and normal skull (at 23 weeks gestation). **b** Type II with hypoplastic thorax with shorts ribs, markedly shortened limbs with straight femurs and a cloverleaf skull (at 22 weeks gestation)

Conclusions

In *FGFR3* molecular prenatal diagnosis, biopsy of the chorionic villi was indicated in cases in which a parent was affected with ACH/HCH. In those cases with a positive molecular study of skeletal dysplasia due to *FGFR3* mutations in a previous pregnancy or in an affected son, a prenatal diagnosis in a following pregnancy may be done for anxiety since germ line mosaicisms must be very rare and have not been reported [21]. In our 19 CVS group, only 7 cases were really indicated for molecular study because there was a positive familial case. Four ACH gravidas, verified by blood samples, were referred for prenatal diagnosis. Two fetuses were positive. The other two lost their fetuses after a difficult transcervical chorionic villus biopsy, both with a negative result. Given their skeletal malformations, the biopsy of chorionic villi is not always feasible and must be considered other invasive technique for prenatal diagnosis. In the study by Gooding *et al.* about prenatal genetic testing for ACH in affected parents, just 38% were interested in the parentally diagnosis of ACH, but participants were not willing to consider termination of pregnancy based on the diagnosis (86%). They were most interested in avoiding the lethal form of ACH (homozygous) when both parents were affected [22]. So it is important to give suitable genetic counselling to choose which invasive technique for prenatal diagnosis is better for the particular case, bearing in mind the psychological impact and the risk of losing the fetus. The current techniques of DNA extraction and the more sensitivity of the molecular tests allows to work

with less DNA concentration so direct AF sample could be a candidate for molecular studies instead of CVS.

The prenatal ultrasonography allows the detection of skeletal dysplasias, but most of them appear in the second trimester or later when the legal termination of pregnancy (TOP) is not possible (until 22 weeks in Spain for foetal anomalies). Accurate prenatal diagnosis of skeletal dysplasias based only on sonographic results is feasible just for the lethal types and can be detected between the 20 and 24 weeks gestation [23–25]. Parrilla *et al.* reported the ratio of femur length-to-abdominal circumference <0.16 with a hypoplastic thorax as indicative of a lethal skeletal dysplasia [17]. In our AF series, micromelia was the most frequent finding by US examination for offering a molecular diagnosis. Just the 4 fetuses which showed short limbs with hypoplastic thorax were positive for a lethal skeletal dysplasia (27%). In 5 CVS cases marked micromelia, with [2] or without [3] a lethal skeletal dysplasia, was the main sign for offering a molecular diagnosis (15 and 20 weeks of gestation). All cases were negative. In the study by Todros *et al.* about the dilemma of the detection of a shortening of femur length (FL), they reported 86 fetuses with a FL below the 10th percentile measured between 15–24 weeks of gestational age, from 1028 pregnant women. There were 28 normal, 18 small-for-gestational age babies and 40 structurally abnormal. Of the 40 malformed fetuses, 16 had chromosomal disorders, 13 had skeletal dysplasias (4 TD, 4 ACH, 1 osteogenesis imperfecta II, and 3 clubfoot) and 11 had other malformations. So, skeletal dysplasias represented the 15% (13/86) of all FL

while chromosomal disorders are more frequent (19%, 16/86) [26], therefore aneuploidy studies are always advisable.

The interpretation of US finding in non-lethal types is even more difficult. ACH have a normal or close to normal femur length until 20–24 weeks. In our series, none of the fetuses with a suspicion of an ACH were positive. Not surprisingly because antenatal diagnosis is difficult as demonstrated by Modaff *et al.*, in their study of children with ACH. From 28 families without previous family history, 12 of these pregnancies were not recognized to have any fetal abnormalities (43%) and in the other 16 cases various diagnoses were provided but none were given a definitive diagnosis of ACH [27]. Reports of prenatally diagnosed HCH fetuses are very rare. The prenatal sonographic detection of HCH is the most difficult due to the absence of specific US markers because many of the subtle symptoms of the disease are not present *in utero* and infancy [21]. The remarkable deviation of the foetal growth curve of femur length from the normal values in conjunction with a normal growth curve of biparietal diameter has been suggested to be a useful US indication for prenatal non-lethal skeletal dysplasia [28]. In those cases, prenatal diagnosis relies first a karyotype and later on an ACH/HCH molecular study.

In tissue abortion is interesting to have a description of the phenotype of the aborted foetus (phenotype TD-I or TD-II) to design the molecular study. Chromosomal abnormalities are the second cause of foetal anomalies so cytogenetic/molecular studies must be done [29, 30]. The malformations constitute the most frequent indication for termination of pregnancy. This group include different pathologies in which skeletal system is a small percentage variable between 6% and 16% [30–32]. Although most of the TA referred to our laboratory came from private centre with no medical or anatomic record, we have diagnosed 30% of TA (5 skeletal dysplasias and 1 triploidy) from 20 cases. Another abortion without molecular study because of deterioration of the sample, had a pathological anatomy study in our hospital indicated a probable 18 trisomy. So, a total of 7 samples have been diagnosed (35%).

Sporadic cases of ACH and other skeletal dysplasia due to mutations in *FGFR3* gene have been demonstrated that occur in the paternally derived chromosome and associated with advanced paternal age [33, 34]. Wyrobek *et al.* studying gene mutations on DNA in advancing male age male, reported a 3.3% increase in ACH mutation frequency per year age [11]. In our group of positive skeletal dysplasias, there are 9 sporadic cases, 6 of them have a paternal age ≥ 35 years (35–43 years), 1 case was 33 years old and in 2 we did not get the information. Due to the high incidence of *de novo* cases, it is recommended high-resolution sonography in weeks 20–22 [10] when the father is older of 35 years old.

Our purpose in this study was to show our experience in molecular studies prenatal diagnosis with suspicion of skeletal dysplasias due to *FGFR3* gene. After a normal karyotype, all exons implicated in the four most common skeletal dysplasias in *FGFR3* gene (ACH, HCH, TD-I, and TD-II) must be studied, obviously starting with the one that phenotype suggests. Molecular diagnosis is always indicated in cases of affected parents, affected son or positive molecular skeletal dysplasias in a previous pregnancy, in both later cases just for anxiety because germ line mosaicism are very rare and have not been reported. A US finding showed hypoplastic thorax with micromelia, above all if the ratio of femur length-to-abdominal circumference < 0.16 , forces to indicate a prenatal diagnosis for molecular study of lethal dysplasias. A protocol for abortion must include an X-ray, pathologic anatomy, and genetic studies. Genetics studies must assess aneuploidy and all known mutations for *FGFR3* gene. There were other skeletal dysplasias with mutations in different genes so it is a best practice to keep a DNA sample for future studies when phenotype suggests another diagnosis and *FGFR3* gene mutations have not been found. Our opinion is that the identification of skeletal dysplasias is quite difficult and requires interdisciplinary cooperation between gynaecology, radiology, pathology and genetics, for a correct diagnosis and therefore providing a better counselling to the parents.

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