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Molecular Analysis of a Case of Thanatophoric Dysplasia Reveals Two de novo FGFR3 Missense Mutations located in cis

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التحليل الجزيئي لحالة خلل التنسج المميت يكشف عن طفرتين مغلوطتين جديدتين لجين FGFR3 تقعان على السيس

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الملخص: **الهدف**: ان خلل التنسج المميت هو أكثر أنواع خلل التنسج الهيكلي القاتل شيوعا وينتج عن صقة جسمية سائدة ويتميز بوجود كبر في حجم الرأس، ضيق في حجم الصدر، أضلع قصيرة، أصابع قصيرة و ارتخاء في العضلات. وبالإضافة إلى هذه الملامح الظاهرية الجوهرية فيتميز النوع الأول من هذا المرض بصغر الأطراف وانحناء عظام الفخذ بينما يتميز النوع الثاني بصغر الأطراف واستقامة عظام الفخذ ووجود تشوه برسيمي في شكل الجمجمة. من المعروف أن كل حالات هذا المرض تنتج عن طفرات في جين ال ف ج ف ر3. هدفت الدراسة الي التعرف على طيف الطفرات المسؤوله عن حدوث هذا المرض. **طرق الدراسة**: تم استخدام تفاعل البلمرة السلسلي التقليدي والأليلي والتحليل التسلسلي لتحديد طفرات جين ال ف ج ف ر3 في جنين مصاب بخالل التنسج المميت والذي تم اكتشافة اثناء الحمل باستخدام الموجات فوق الصوتية. **النتائج**: في هذا البحث نستعرض كيفية تحديد طفرتين مغلوطتين جديدتين لجين ال ف ج ف ر3 تقعان على السيس في جنين لديه العلامات الظاهرية لمرض خلل التنسج المميت. **الخلاصة**: هذه هي المرة الثانية التي يتم فيها وصف حالة خلل التنسج المميت نتيجة لوجود طفرتين مغلوطتين جديدتين لجين ال ف ج ف ر3 تقعان على السيس مما يدل على أن طيف الطفرات المسببة لهذا المرض قد يكون اكثر شيوعا مما هو معتقد.

مفتاح الكلمات: خلل التنسج المميت، جين ف ج ف ر3، خلل التنسج الهيكلي، تفاعل البلمرة السلسلي الأليلي.

ABSTRACT: ***Objectives:*** Thanatophoric dysplasia (TD) is the most common form of lethal skeletal dysplasia. It is primarily an autosomal dominant disorder and is characterised by macrocephaly, a narrow thorax, short ribs, brachydactyly, and hypotonia. In addition to these core phenotypic features, TD type I involves micromelia with bowed femurs, while TD type II is characterised by micromelia with straight femurs and a moderate to severe clover-leaf deformity of the skull. Mutations in the *FGFR3* gene are responsible for all cases of TD reported to date. The objective of the study here was to delineate further the mutational spectrum responsible for TD. ***Methods:*** Conventional polymerase chain reaction (PCR), allele-specific PCR, and sequence analysis were used to identify *FGFR3* gene mutations in a fetus with a lethal skeletal dysplasia consistent with TD, which was detected during a routine antenatal ultrasound examination. ***Results:*** In this report we describe the identification of two *de novo* missense mutations in *cis* in the *FGFR3* gene (p.Asn540Lys and p.Val555Met) in a fetus displaying phenotypic features consistent with TD. ***Conclusion:*** This is the second description of a case of TD occurring as a result of double missense *FGFR3* gene mutations, suggesting that the spectrum of mutations involved in the pathogenesis of TD may be broader than previously recognised.

Keywords: Thanatophoric dysplasia; FGFR3; Skeletal dysplasia; Allele-specific PCR.

Advances in Knowledge

- *This study is only the second report in which the mutations in the FGFR3 gene differ from those classically implicated in the development of thanatophoric dysplasia (TD).*

Applications to Patient Care

- *The findings suggest that confirmation of a clinical diagnosis of thanatophoric dysplasia should include a comprehensive screen for mutations in all coding exons of the FGFR3 gene, particularly in the absence of one of the classic TD mutations.*

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Thanatophoric dysplasia (TD) is the most common form of lethal short-limb skeletal dysplasia with a prevalence of approximately 2/100,000 births.[1] It was first described as thanatophoric dwarfism by Maroteaux and Lamy in 1967, undergoing a name change in 1977 as a result of a decision reached at the 2nd International Conference on the Nomenclature of Skeletal Dysplasias.[2] It is characterised by a range of phenotypic features, foremost among which are macrocephaly, a narrow thorax, short ribs, brachydactyly, and micromelia.[3] There are two forms of TD, designated TD type I and TD type II, which can be differentiated principally by the presence of bowed, rather than straight, femurs in TD type I and of a moderate to severe clover-leaf deformity of the skull in TD type II.[3] A clover-leaf skull deformity may also occur in TD type I but is infrequent and less severe than in TD type II.[3] Both subtypes of TD are considered lethal with most affected infants dying as a result of respiratory insufficiency within the first few hours of life; however, there are reports of a small number of affected individuals who have survived into childhood by virtue of aggressive ventilatory support.[4]

As with the other severe skeletal dysplasias, TD is frequently diagnosed following a routine antenatal ultrasound screening in the first or second trimester. The earliest radiological signs are increased nuchal thickness and short limbs, but these signs are non-specific.[5,6] A molecular diagnosis may be made prenatally if genetic testing for TD is requested on deoxyribonucleic acid (DNA) extracted from a chorionic villus or amniocentesis sample, but is more commonly made following a post-mortem examination carried out to clarify the nature of the lethal skeletal dysplasia in question.[5,6]

TD is primarily an autosomal dominant disorder, resulting from heterozygous mutations in the *FGFR3* gene that lead to ligand-independent activation of the receptor and disruption of cartilage function during linear bone growth.[7] Mutations in the *FGFR3* gene cause a spectrum of skeletal dysplasias ranging from the relatively mild hypochrondroplasia through to achondroplasia, severe achondroplasia with developmental delay and *acanthosis nigricans* (SADDAN), and thanatophoric dysplasia.[7] As expected from the severity of the disorder, the majority of TD-related mutations in the *FGFR3* gene are *de novo* and the recurrence risk for parents with a previously affected pregnancy is very low, particularly if both parents are phenotypically normal.[3] A single case of somatic and germline mosaicism for the TD type 1 mutation Arg248Cys has been described.[8] The mosaic individual in this instance was affected by a disproportionate asymmetric rhizomelic shortening of the limbs. Her only pregnancy was complicated by severe pre-eclampsia and resulted in the stillbirth of an infant with a phenotype consistent with TD.[8]

We present here a case in which TD was diagnosed prenatally by ultrasound scanning and describe the results of subsequent molecular analysis, which revealed a double *de novo* mutational event.

Methods

Informed consent was provided by the parents of the proband reported here; the proband was studied at the genetic level to confirm a clinical diagnosis of TD. The New Zealand multi-region ethics committee has ruled that cases of patient management do not require formal ethics committee approval.

A skin biopsy from the affected fetus was taken at autopsy and the tissue was cultured according to conventional cytogenetic techniques. Genomic DNA (gDNA) was isolated from the cultured cells by phenol/chloroform extraction and ethanol precipitation. In the case of peripheral blood in ethylenediaminetetraacetic acid (EDTA), gDNA was isolated using the Gentra Puregene DNA Extraction kit (Qiagen Pty Ltd, Doncaster, Australia).

The messenger ribonucleic acid (mRNA) sequence of transcript 1 of the *FGFR3* gene was identified using the University of California Santa Cruz (UCSC) genome browser.[9] This website provides a direct link to ExonPrimer for the design of primers flanking coding exons. The first coding exon of transcript 1 of the *FGFR3* gene is exon 2, so primers were not designed against exon 1. ExonPrimer is a Perl script that uses a combination of Primer3 and Blat to design intronic primers against a complementary DNA (cDNA) of interest. Default parameters were used. The coverage (entire coding exon and at least 20 bp of flanking intronic sequence to allow interrogation of splice sites) and specificity of the primers were confirmed using the *in-silico* polymerase chain reaction (PCR) tool also

Table 1: Primers for the amplification of all coding exons of the *FGRF3* gene (Refseq accession number NM_000142.4)

Exon	Forward primer	REVERSE PRIMER	Amplicon size (bp)
2	TGTAAAACGACGGCCAGTGAGCTGCCTTC CTCCTCC	CAGGAAACAGCTATGACCCGGGGCGTCA CTCACAC	380
3	TGTAAAACGACGGCCAGTGCTGTGTCTGT AAACGGTGC	CAGGAAACAGCTATGACCGACCCACGCA GGGACTC	441
4	TGTAAAACGACGGCCAGTTCTCTCTGGTC ATTGGTGGA	CAGGAAACAGCTATGACCAGCCCCTCCTG TATCCTGAG	394
5	TGTAAAACGACGGCCAGTTCCTACACAGG ACGGGAAAC	CAGGAAACAGCTATGACCGGATGCTGCCA AACTTGTTC	507
6	TGTAAAACGACGGCCAGTCCATCTCCTGG CTGAAGAAC	CAGGAAACAGCTATGACCTGCGTCACTGT ACACCTTGC	500
7	TGTAAAACGACGGCCAGTTGGACGTGCT GGGTGAG	CAGGAAACAGCTATGACCCAACCCCTAGA CCCAAATCC	433
8	TGTAAAACGACGGCCAGTTGTGGACTCTG TGCGGTG	CAGGAAACAGCTATGACCCTTTGGCGTG TCCCGAG	316
9	TGTAAAACGACGGCCAGTCTCCCAGTGG TGCCTGC	CAGGAAACAGCTATGACCAGAGAGGGCTC ACACAGCC	422
10	TGTAAAACGACGGCCAGTCTTCATTCAAT GCTGGTGGA	CAGGAAACAGCTATGACCACAGAACCCCA GCCACAC	436
11	TGTAAAACGACGGCCAGTGAGCATGGAG GGCTTCCT	CAGGAAACAGCTATGACCTTTCCCGATCA TCTTCATCA	436
12	TGTAAAACGACGGCCAGTGCCAAGCCTGT CACCGTAG	CAGGAAACAGCTATGACCACACCAGGTCC TTGAAGGTG	487
13-14	TGTAAAACGACGGCCAGTGGTAGGTGCG GTAGCGG	CAGGAAACAGCTATGACCCCAGGCGTCC TACTGGC	594
15-16	TGTAAAACGACGGCCAGTGGGGTCATGC CAGTAGG	CAGGAAACAGCTATGACCTATTCGGGAAC AGCCTGAAG	619
17-18	TGTAAAACGACGGCCAGTCAGGCTGTTCC CGAATAAGG	CAGGAAACAGCTATGACCCACCAGCAGC AGGGTGG	589

Blue and red coloured bases represent M13 sequences that tail the forward and reverse primers.

available on the UCSC genome browser. All primers were checked for single nucleotide polymorphisms using the software tool available from the National Genetic Reference Laboratory, Manchester, UK.[10] The primers were tailed with M13 sequences for ease of subsequent sequence analysis, and were synthesised by Invitrogen Ltd., (Renfrewshire, UK) [Table 1].

We used the freely accessible online tetra-primer amplification refractory mutation system (ARMS)-PCR primer design programme[11] to design allele-specific PCR primers to target the DNA variants of interest.[12] Specificity is ensured by not only using allele-specific primers with differing 3' terminal bases but also including an additional mismatched base at position -2 from the 3' end. Two pairs of primers were designed. Each pair contained one allele-specific primer that would result in amplification of only the wild-type allele of each of the two variants in question. The other primer in each pair was positioned such that the second variant was also included within the amplicon, thereby allowing the phase of the variants to be determined. These two additional primer pairs were likewise tailed with M13 sequences and were synthesised by Invitrogen Ltd. (Renfrewshire, UK).

Conventional and allele-specific PCR were performed using 1U Faststart Taq DNA polymerase (Invitrogen Ltd, Renfrewshire, UK), 50 ng genomic DNA, 2mM MgCl2, and 0.8 µM forward and reverse primers with the following cycle conditions: 95° C for 4 minutes, 35 cycles of 94° C for 45 seconds, 60° C for 30 seconds, 72° C for 30 seconds, and a final extension at 72° C for 10 minutes. All amplicons amplified efficiently under these conditions.

An amount of 5 µL of each PCR was cleaned with

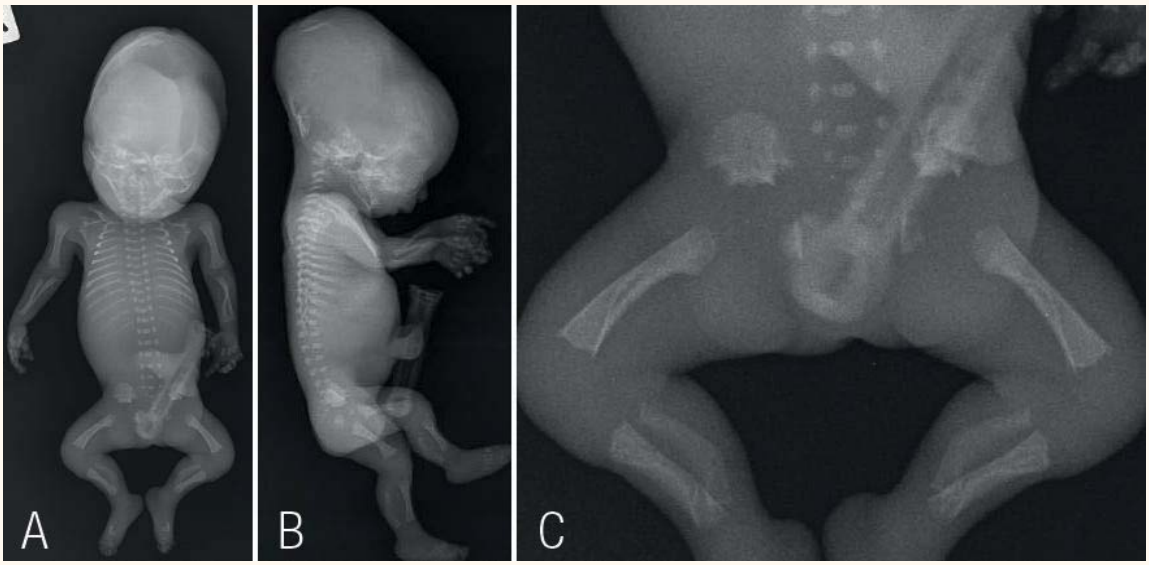

Figure 1: Fetal phenotype: skeletal radiographs of anteroposterior (I), lateral (II) and pelvic (III) views.

ExoSAP-IT (Affymetrix, Santa Clara, California, USA) prior to bidirectional DNA sequencing using M13 forward and reverse primers and Big-Dye Terminator, Version 3.0 (Applied Biosystems Ltd., Carlsbad, California, USA). An amount of 20 µL of sequenced product was purified using an automated Agencourt CleanSEQ (Agencourt Bioscience Corp., Beverly, Massachusetts, USA) procedure with the aid of an epMOTION 5075 liquid handling robot (Eppendorf, Hamburg, Germany). An amount 15 µL of purified product was then subjected to capillary electrophoresis using an Applied Biosystems model 3130xl genetic analyser.

The analysis of sequence traces was performed using Variant Reporter, Version 1.0 (Applied Biosystems). Genebank NM_000142.4 was used as the reference sequence, with cDNA number +1 corresponding to the A of the translation initiation codon. Amino acid numbering began at the first amino acid in RefSeq accession number NP_000133.1. Exons were numbered sequentially, with exon 2 being the first exon analysed (exon 1 is non-coding). Variant Reporter uses advanced algorithms and quality metrics to automate the detection of variants and to streamline the analysis process. Traces produced from the sequencing of allele-specific PCR amplicons were analysed manually.

Results

The fetus was the third child to non-consanguineous parents. The first antenatal ultrasound scan at 13 weeks gestation demonstrated a nuchal thickness of 2.0 mm. The second ultrasound at 19 weeks was technically very difficult because of fetal lie and maternal habitus. However, it demonstrated the presence of a grossly enlarged skull with an abnormal shape (biparietal diameter [BPD] 59 mm, head circumference [HC] 207 mm). The fetal chest was subjectively small and the limbs were short with both femoral length (FL) and humeral length (HL) well below the 5th centile (FL 22 mm; HL 19 mm). These findings were indicative of a lethal skeletal dysplasia. The parents were counselled accordingly and decided to proceed with a termination which was performed at 19 weeks and 5 days' gestation.

Skeletal radiographs [Figure 1] showed a narrow thorax in both anteroposterior and lateral views. The skull was large in relation to the facial bones and abnormally shaped but without a clover-leaf appearance. The vertebral bodies were flat with an H-shaped appearance but no notching of the central portions of the upper and lower plates. There was narrowing of the lumbar interpediculate distances from L1–L3. The long bones were short and relatively broad with mild bowing of the femora. The metaphyseal regions were cupped and flared. The pelvic bones were short and broad with horizontal inferior margins of the iliac bones.

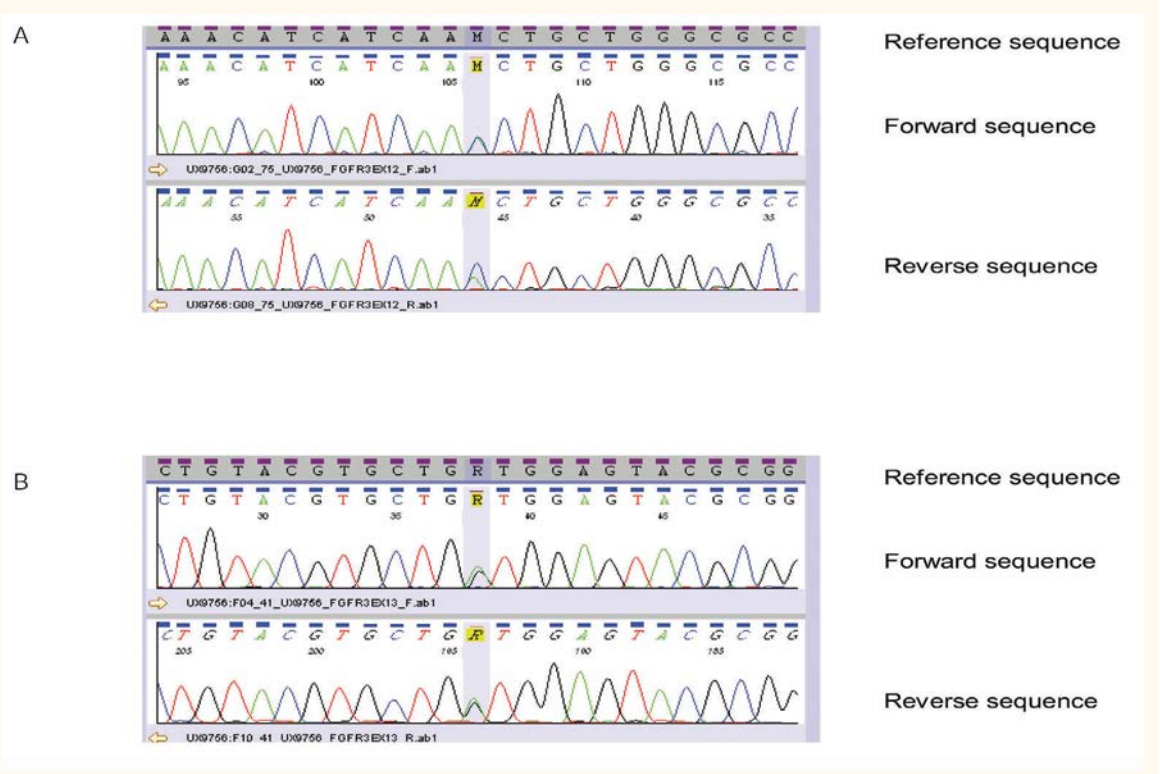

Figure 2: (**Panel A**) Fetal genotype: sequence electropherograms show the c.1620C>A (p.Asn540Lys); (**Panel B**) c.1663G>A (p.Val555Met) variants in exons 12 and 13, respectively, of the *FGFR3* gene in the affected fetus.

Overall, the radiologic findings were consistent with TD with an overlap between TD type I and TD type II. The vertebral abnormalities were more consistent with TD type II, while the mild femoral angulation was not characteristic of TD type II, but was not as severe as would be expected in TD type I.

Conventional PCR and bidirectional sequence analysis of all coding exons of the *FGFR3* gene in the affected fetus detected compound heterozygosity for the mutations c.1620C>A (p.Asn540Lys) and c.1663G>A (p.Val555Met) in exons 12 and 13, respectively, of the *FGFR3* gene [Figure 2].

In order to determine the phase of these mutations, subsequent allele-specific PCR was performed [Figure 3]. Sequencing of the amplicons produced by allele-specific PCR demonstrated that the variants were present in *cis* [Figure 4].

DNA extracted from the peripheral blood of both parents was analysed for the presence of each of these variants. Both variants proved to be absent in each parent, indicating that they were most probably *de novo* in the fetus (gonadal mosaicism in one of the parents has not been excluded).

Discussion

The case we present here exhibits the classic phenotypic features of TD with overlapping features of type I and type II. Molecular genetic testing for TD type I involves either full sequence analysis of all coding exons of the *FGFR3* gene (as was the approach taken in this case), or more targeted initial analysis of exons 7, 10, 15, and 19, which are the exons containing the mutations responsible for 99% of cases of TD type I reported to date. This is followed by full analysis if no mutation is identified.[3] The mutations themselves can be placed into two classes. The first includes the two most common TD type I mutations, Arg248Cys and Tyr373Cys, and involves the creation of new unpaired cysteine residues.[3] The second class of pathogenic TD type I mutations result in obliteration of the native stop codon and addition of a hydrophobic alpha helix-containing domain to the carboxyl terminus of the protein.[3] The single mutation known to cause TD type II, p.Lys650Gly, destabilises the activation loop of the second portion of the split tyrosine kinase domain resulting in ligand-independent activation.[11]

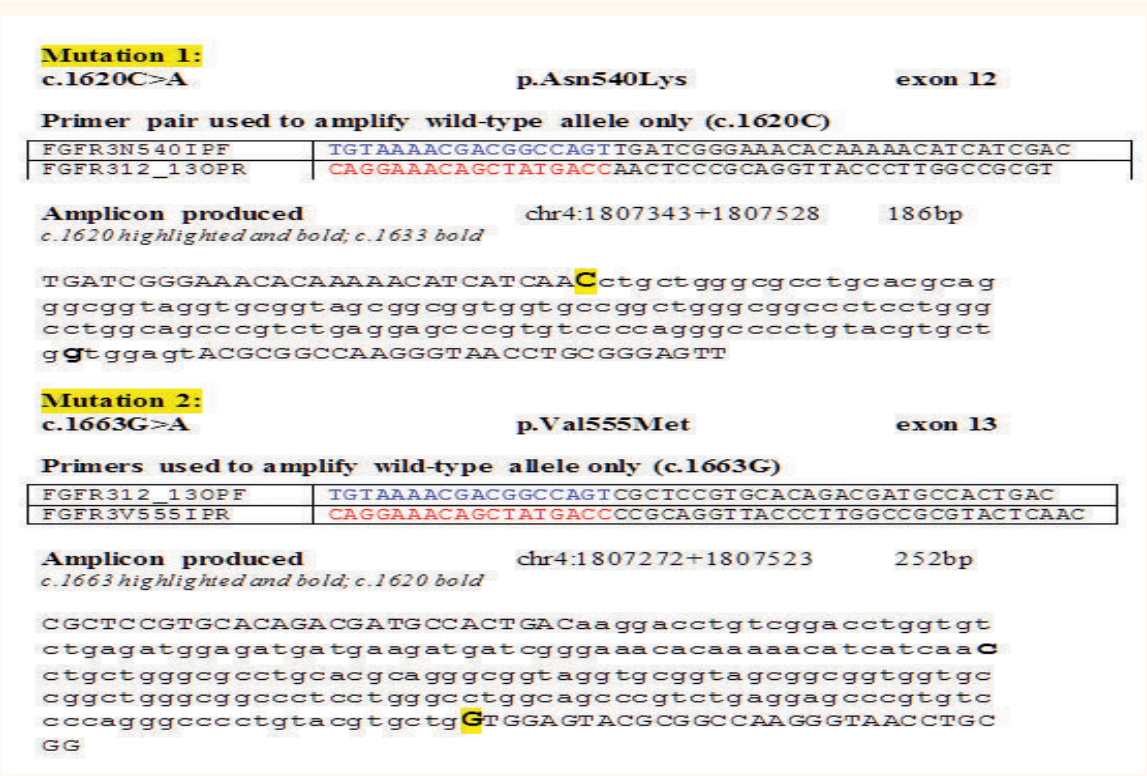

Figure 3: Sequences and primers for allele-specific PCR: allele-specific primer sequences are shown to determine the phase of the *FGFR3* gene mutations detected in the affected fetus.

Neither of the mutations detected in the fetus described above fall into these categories. The first variant, c.1620C>A (p.Asn540Lys), is present in the heterozygote state in approximately 72% of individuals with classic hypochondroplasia.[14] The second variant, c.1663G>A (p.Val555Met), is not listed in the online Human Gene Mutation Database (HGMD) and does not appear to be reported in the literature. Bioinformatic analysis using the online prediction tools Polyphen-2[15] and SIFT[16] suggests that it is highly likely to be of pathogenic significance. In addition, valine at position 555 is conserved across species.

Compound heterozygosity in *trans* for the Asn540Lys mutation and the Gly380Arg mutation that is responsible for 98% of cases of achondroplasia[17] has been reported.[18–21] Individuals who are compound heterozygotes for these two variants typically display a more severe skeletal phenotype than seen in classical achondroplasia, but the condition is still compatible with survival, unlike the lethal form of achondroplasia that results from homozygosity for the Gly380Arg mutation. It has been shown that the level of ligand-independent tyrosine kinase activity is directly related to the skeletal phenotype, so it is to be expected that the combination of the less-activating mutation Asn540Lys and Gly380Arg will result in a less severe phenotype than that caused by homozygosity for Gly380Arg alone.[13]

In contrast, a case of apparent TD type I in association with compound heterozygosity for Asn540Lys and a second previously unreported mutation, Gln485Arg, has also been described.[22] In this instance, as in our case, the two *FGFR3* mutations identified were *de novo* in the affected fetus and in *cis*. Analysis of the deduced X-ray crystallographic structure of *FGFR3* led the authors to postulate that the presence of two mutations in *cis* alters the receptor structure to such an extent that it is held in a fully activated state, with each mutation attenuating the effect of the other, leading to the lethal form of skeletal dysplasia characteristic of TD.[22]

Each of the mutations identified in our fetus is located within the first portion of the split intra-cellular tyrosine kinase domain.[23] It has been recognised that the activation of *FGFR3* caused by substitutions within the tyrosine kinase domain is due to a mechanism that mirrors the conformational changes that are normally a result of ligand-mediated *FGFR3* dimerisation and

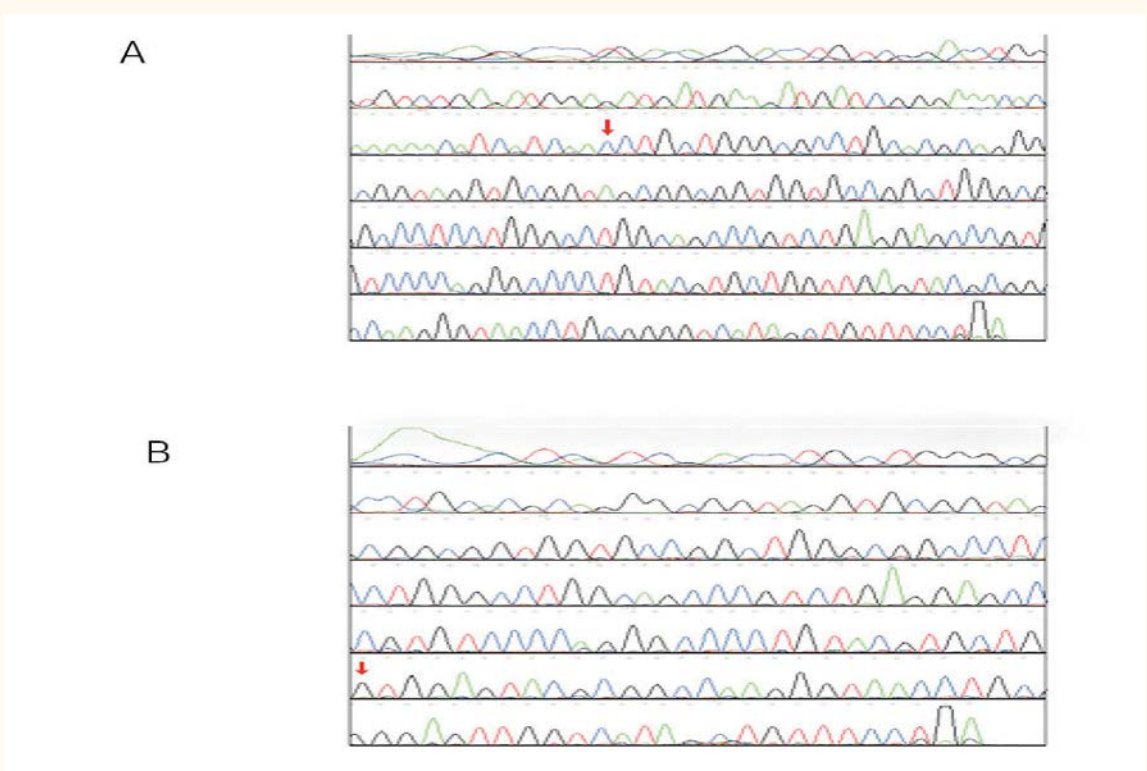

Figure 4: Sequence electropherograms of allele-specific PCR. (**Panel A**)Amplicon produced by primers specific for wild-type allele at c.1663. The red arrow indicates position c.1620—note the apparent homozygosity for wild-type c.1620C. (**Panel B**) Amplicon produced by primers specific for wild-type allele at c.1620. The red arrow indicates position c.1663—note the apparent homozygosity for wild-type c.1663G.

autophosphorylation.[23,24] Double mutation alleles have been reported in association with a range of disorders, often complicating genotype-phenotype correlations.[25,26] It is probable that the basis of the severe phenotype seen in our case, in contrast to the phenotype that results from the Asn540Lys mutation alone or in combination with Gly380Arg, is related to the 'double-hit' effect of two mutations both acting in this critical region of the protein. It is the combination of the location of the mutations and the attenuating effect they have upon each other that is significant.

A recent review of the current understanding of *FGFR3* signalling in the skeletal dysplasias concludes that, although progress is being made, there are still a large number of questions to be answered regarding almost every aspect of the action of *FGFR3*, particularly when it comes to cartilage.[23] The rate of *de novo* double mutations occurring in the same gene in *cis* is predicted to be very low at approximately,[12–13] therefore it is unlikely to be a common mechanism in the pathogenesis of TD.[27]

Conclusion

The case we describe is the second report in which the mutations differ from those classically implicated in the development of TD. Findings such as this may be usefully incorporated into the design of functional studies in the future, and suggest that the range of genotypes potentially responsible for the characteristic TD phenotype is likely to be broader than previously recognised.

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