**Non-invasive Prenatal Diagnosis for Thanatophoric Dysplasia**

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**BACKGROUND AND OBJECTIVES**

Cell-free fetal DNA (cfDNA) is an alternative source of fetal genetic material that can be used for non-invasive prenatal diagnosis (NIPD). It can be detected from seven weeks gestation, and usually constitutes <10% of cell-free DNA in maternal plasma; the majority being maternal in origin. Thanatophoric dysplasia (TD) is a lethal autosomal dominant skeletal dysplasia caused by de novo mutations in the fibroblast growth factor receptor 3 (FGFR3) gene. TD is classified into 2 subtypes (TD1 and TD2) based on characteristic skeletal features. Accurate diagnosis of skeletal dysplasias using ultrasound is challenging; molecular diagnosis is the more reliable approach.

Our objective was to develop non-invasive prenatal diagnosis (NIPD) using cell-free fetal DNA (cfDNA) for thanatophoric dysplasia.

**METHODS**

**Plasma Preparation**

20ml of blood was taken into EDTA tubes from women with at-risk pregnancies based on a previous affected pregnancy or abnormal ultrasound findings. Blood was spun within 6 hours of collection and plasma extracted; alternatively blood was stored at -80°C until processing.

**DNA Extraction**

Cell-free DNA (cfDNA) was extracted from 5ml plasma samples using the QiaSep Circulating Nucleic Acid™ kit according to manufacturers instructions into a final volume of 75µl AVE elution buffer.

**PCR**: Restriction Digest

Primer sets TD1F and TD1R were used to amplify the segment of DNA containing nucleotide c.742. Two sets of primers (TD1F1P and TD1R1, and TD1F2 and TD1R2) were used to amplify the region containing nucleotide c.1948. 10µl of plasma DNA or 50ng of gDNA was used as a template for each PCR, using touchdown PCR. For detection of the c.1948A>G mutation, 5µl of Blau enzyme was used to digest 25µl of PCR product at 37°C for two hours. For detection of the c.742C>T transition 10U of AfeI, BsiHKAI and DraIII enzymes were used to digest 25µl of DNA at either 37°C (AfeI and DraIII) or 65°C (BsiHKAI) for two hours. In all cases where the mutation analysis was negative, the plasma DNA was tested using quantitative real time PCR for SKY or a panel of baldrick indel polymorphisms to confirm the presence of cfDNA.

**Digital PCR**

Primer and MGB hydrolysis probes to detect the c.742C>T, c.746C>G (both type I TD) and c.1948A>G (type II TD) mutations were designed using Primer Express Software. One probe in each case was FAM-labelled and another VIC-labelled probe (indicated in red) and mutant sequences by a VIC-labelled probe (blue). The test cffDNAs panels are indicated with arrows; in both cases there are only wild type sequences present, indicating that both fetuses are unaffected. A cfDNA sample from a fetus known to be affected by the TD mutation was used as a positive control (shown in panels 7 and 8). Genomic DNA from maternal blood was used to control for maternal cell contamination, and was negative for the mutation in all cases. cfDNA from an unaffected pregnancy was used as a negative control (panels 5 and 6).

**RESULTS**

- **Normal Control**
  - TD plasma
  - Normal
  - TDI
  - TDI
  - Normal
  - TDI

**Fig. 1** Sonographic findings in two fetuses with confirmed Thanatophoric Dysplasia

**Fig. 2** Restriction analysis of PCR products for two thanatophoric dysplasia mutations. (a) The c.742C>T type I TD mutation was detected using PCR followed by digestion with AfeI, BsiHKAI and DraIII. cfDNA from a woman carrying an unaffected fetus is digested with AfeI (lane 1) but remains uncut using BsiHKAI (lane 4) and DraIII (lane 5); conversely in an affected fetus the AfeI site is destroyed leaving some of the cfDNA undigested (lane 7) and a BsiII site (lane 8) and a DraIII site (lane 9) are created. (b) The c.1948A>G mutation was detected using digestion with the BsiII enzyme and less different primer sets. In the presence of an unaffected fetus, all cfDNA is digested by BsiII (lanes 3 and 8), whereas with an affected fetus, the BsiII restriction site is destroyed, leaving some of the cfDNA undigested (lanes 5 and 11).

**Fig. 3** Digital PCR for the c.742C>T mutation. Wild type sequences were detected using a FAM-labelled probe (indicated in red) and mutant sequences by a VIC-labelled probe (blue). The test cffDNAs panels are indicated with arrows; in both cases there are only wild type sequences present, indicating that both fetuses are unaffected. A cfDNA sample from a fetus known to be affected by the TD mutation was used as a positive control (shown in panels 7 and 8). Genomic DNA from maternal blood was used to control for maternal cell contamination, and was negative for the mutation in all cases. cfDNA from an unaffected pregnancy was used as a negative control (panels 5 and 6).

**Fig. 4** Summary of the TD NIPD analysis carried out. One result (case 9) was inconclusive using PCR-RED, but was shown to be positive using digital PCR, and this result was confirmed using fetal skin at post-mortem. Case 2 appeared negative by PCR-RED and we had no cfDNA left to test with digital PCR. USs – ultrasound scan; PIH indicates past history; GA – gestational age; cfDNA shows the result using PCR-RED; n/d – not determined; PM tissue – post-mortem tissue.

**CONCLUSIONS**

- NIPD can be used for the accurate diagnosis or exclusion of Thanatophoric Dysplasia.
- Based on our preliminary results, the PCR-RED test sensitivity is 86% (6/7) with a specificity of 100% (4/4).
- PCR-RED can be used to test for the most common Type I and Type II mutations; however, digital PCR is more sensitive than PCR-RED allowing detection of mutations at a lower cfDNA concentration.

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