Pregnancy following preimplantation genetic diagnosis for Crouzon syndrome

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Crouzon syndrome is a dominantly inherited craniosynostosis syndrome which is caused by mutations in the fibroblast growth factor receptor 2 gene (FGFR2). However, a specific point mutation in the FGFR3 gene has also been shown to result in Crouzon syndrome associated with acanthosis nigricans. We report here the first method for preimplantation genetic diagnosis (PGD) of Crouzon syndrome based on multiplex PCR amplification followed by the direct detection of the causative mutation by single-stranded conformational polymorphism (SSCP) analysis. A highly polymorphic short tandem repeat (STR) locus was simultaneously analysed as a control against some forms of contamination. The mutation, carried by the female partner, was a de-novo substitution at codon 338 of the FGFR2 gene. The couple were found to be informative at the D21S11 STR locus. Two clinical PGD cycles were performed, resulting in the biopsy of 36 blastomeres, 25 of which showed amplification at the FGFR2 locus. All of the cells showed expected genotypes at the D21S11 locus with only one incidence of allele drop-out. A total of five embryos were transferred, two in the first cycle and three in the second, resulting in a singleton pregnancy.

Key words: Crouzon syndrome/FGFR2/PGD/preimplantation genetic diagnosis/single cell PCR

Introduction

Craniosynostosis is the most common congenital defect of the skull in humans and has a birth prevalence of 1 in 2000–3000 live births (Lajeunie et al., 1995). Over 100 syndromes associated with craniosynostosis have been delineated, the majority of which exhibit dominant inheritance (Winter and Baraitser, 1996). The clinical classification of these syndromes depends on the presence of specific craniofacial abnormalities, which occur with or without specific limb involvement. Historically, Crouzon syndrome has been distinguished clinically by an absence of limb malformations, although more recent radiological examinations of patients have revealed frequent subtle malformations (Anderson et al., 1997).

To date, patients diagnosed with Crouzon syndrome, and in whom a causative mutation has been identified, have all been shown to carry mutations of the fibroblast growth factor receptor 2 (FGFR2) gene (Reardon et al., 1994). However, pathogenic mutations remain to be identified in ~50–73% of patients, and these may be due to genetic heterogeneity which has been observed in other craniosynostosis syndromes such as Apert and Pfeiffer. Alternatively, the mutations may be situated outside the coding region for the immunoglobulin-like III (IgIII) domain and the linker region connecting this loop with the immunoglobulin-like II (IgII) domain, where the majority of mutations identified to date are situated (Mulliken et al., 1999; Passos-Bueno et al., 1999). In addition to ‘classic’ Crouzon syndrome, a single base substitution at codon 391 (A→E) of FGFR3 results in Crouzon syndrome associated with acanthosis nigricans, a hyperkeratotic skin disorder with hyperpigmentation. Crouzon syndrome has an estimated birth prevalence of 15–16 in $10^6$ births (Cohen and Kreiborg, 1992).

The spectrum of mutations in FGFR2 is relatively limited compared with other genes. To date only 46 mutations have been identified, the majority of which are missense, with a smaller number of splice mutations or small insertions/deletions, all of which remain in-frame. A mutation ‘hot spot’ has been identified in the coding region of the IgIII domain (Muenke and Schell, 1995). The three highest germline point mutation rates described in humans all concern FGFR genes. Mutation rates elevated 1000-fold over the background rate have been described at two codons (G380R and P250R) in FGFR3 (Bellus et al., 1995; Moloney et al., 1997) and at one codon (S252W) in FGFR2 (Moloney et al., 1996). Increased paternal age and exclusive paternal origin of de-novo mutations has been described in studies of craniosynostosis families (Risch et al., 1987; Moloney et al., 1996), implicating
spermatogenesis in the elevated mutation rate. However, the mechanisms involved have not yet been elucidated.

The other noteworthy observation is that the 46 FGFR2 mutations result in seven distinct phenotypes, some of which, such as Apert and Pfeiffer syndrome, also arise due to mutations of FGFR1 and 3. Genotype–phenotype correlations have been identified; however, few are absolute. Wide phenotypic variability for a given mutation has been observed between and even within families. Furthermore, reports have suggested that different syndromes may be caused by identical mutations (Gorry et al., 1995). As the majority of the mutations identified to date in the FGFR genes are predicted to alter the immunoglobulin domains, the causes underlying the extraordinarily high amount of heterogeneity and phenotypic variability observed may be due to variable expression of the receptor itself or differences in the dimerization or heterodimerization of the receptor (Mulliken et al., 1999). It is also possible that other developmental genetic factors may be involved (Passos-Bueno et al., 1999).

We describe here the first protocol optimised for the detection of a single base pair substitution in the FGFR2 gene at the single cell level. The presented method involves DNA amplification using a multiplex nested PCR, for the simultaneous amplification of a fragment of the FGFR2 gene encompassing the mutation and a highly polymorphic short tandem repeat (STR) locus located on chromosome 21. Single-stranded conformation polymorphism (SSCP) was then used to detect the mutation. The genotype of the embryos at the STR was determined by fragment analysis on an automated DNA sequencer by fluorescent polyacrylamide gel electrophoresis (PAGE). The STR was included in the assay as a means of detecting extraneous and parental cell contamination. As it was not linked to the mutation, it could not be used to infer the presence or absence of the mutation. The inclusion of linked polymorphisms in single cell diagnoses of dominant disorders can reduce the risk of misdiagnosis due to allele drop-out (ADO) (Wells and Sherlock, 1998). ADO is defined as the random amplification failure of one allele, and as such can only be observed in heterozygous cells. Two intragenic single nucleotide polymorphisms (SNP) had been described in FGFR2 at the time of preparation for the diagnosis (Reardon et al., 1994). However, neither was suitable for use in this case, as the parents were uninformative. Additionally, none of the published STR markers in the vicinity of the gene could be used in the diagnosis as they were all >1 cM away. At that distance the possibility of a recombination event occurring between the marker and the site of the mutation cannot be discounted. Therefore, to reduce the risk of misdiagnosis due to ADO, embryos would only be considered for transfer if results were available from two cells.

Materials and methods
The presented work was carried out under licence from the Human Fertilisation and Embryology Authority (HFEA) and with the approval of the University College London Hospitals Research Ethics Committee.

Patient description
The causative mutation was identified in the female partner as a de-novo G/A single base pair substitution at codon G338E situated in the alternative coding domain for the 3’ half of the FGFR2 IgIII domain. At the time of treatment, the patient was 34 years old and of proven fertility, having conceived an affected girl who subsequently died aged 18 months during corrective surgery. The patient was diagnosed with Crouzon syndrome at 16 years of age and is mildly affected.

IVF
ICSI was used to fertilize all oocytes collected following standard ovarian hyperstimulation protocols (Runieri et al., 2001). Prior to fertilization, the cumulus and corona radiata cells were removed by a combination of enzymatic and mechanical procedures. Only oocytes which had matured to the metaphase II stage were selected for fertilization.

Sample preparation and cell lysis
Buccal cells were obtained by scraping the inside of the cheek with a sterile wooden spatula and suspended in 3 ml phosphate-buffered saline (PBS; Gibco BRL, Paisley, UK) containing 4% (v/v) bovine serum albumin (BSA) (Süttérlin et al., 1999). Blastomeres used for clinical diagnoses were biopsied from cleavage stage embryos. Zona drilling was performed using acidified Tyrode’s solution. Embryo biopsy was carried on day 3 post-fertilization and, where possible, two blastomeres were biopsied per embryo. However, in general only one blastomere was taken from embryos which consisted of five cells or less. All embryos were incubated in Ca2+-/Mg2+-free medium (Scandinavian IVF, Saffron Walden, Essex, UK) for 5 min prior to biopsy in order to facilitate the procedure.

Embryos diagnosed as affected or those donated for research were disaggregated by repeated pipetting following the removal of the entire zona pellucida by incubation in acidified Tyrode’s solution. Single cells were isolated under an inverted microscope using finely drawn, polished micro-capillaries. All cells were washed three times in droplets of PBS/BSA before their transfer in 2 µl PBS/BSA to microcentrifuge tubes containing 3 µl of 125 µg/ml proteinase K and 4×10−4% (w/v) sodium dodecyl sulphate. Cells were lysed by incubation at 37°C for 1 h and the proteinase K was inactivated for 15 min at 95°C.

PCR
Reaction mixes were added to the template in a final volume of 25 µl. The final concentrations per reagent were: 25 pmol each primer [forward outer primer, mutation locus 5’-CTAGGCCGCGGT-3’], reverse primer, mutation locus 5’-CTTGCGC-AGAACTGTCAACCATGCAG-3’ and D21S11 primers as previously described (Sharma and Litt, 1992)], 0.2 mmol each dNTP, 1× Taq buffer and 2 U Taq polymerase (HT Biotechnology, Cambridge, UK). Thermal cycling consisted of an initial denaturation step of 94°C for 4.5 min followed by 37 cycles for the outer reaction and 25 cycles for the inner reaction of 94°C for 30 s, 61°C (outer) and 63°C (inner) for 45 s, 72°C for 45 s, and a final extension step of 72°C for 10 min. The hemi-nested PCR was performed on 1 µl aliquots taken from the first reaction in a fresh reaction mix containing the inner set of primers (forward nested primer, mutation locus 5’-GGGGCAATTCGTGAACTAGAGCTTG-3’, and the reverse primer, mutation locus as described above). All other reagents in the mix were as previously described. Stringent counter-contamination procedures were implemented as previously described (Süttérlin et al., 1999).
Mutation detection

The mutation was detected by silver-stained SSCP on the semi-automated PhastSystem® electrophoresis system using 20% polyacrylamide gels at 4°C. The patients were also genotyped at two intragenic single nucleotide polymorphisms by SSCP; a G/A in intron 7 (~133 nucleotides from exon 8) on the ALFexpress® (Pharmacia, Little Chalfont, Buckinghamshire, UK) at 15°C using 0.5% MDE gel (Flowgen, UK) and a G/A in intron 8 (~575 nucleotides from exon 8) on the GenePhor® system (Pharmacia) at 15°C using a 12.5% polyacrylamide gel. Fluorescently-labelled D21S11 locus PCR products were sized by electrophoresis through a 6% denaturing polyacrylamide gel at 38°C on an automated DNA sequencer (ALFexpress; Pharmacia). Included in each lane were two size standards flanking the product, allowing the sizing of fragments up to 1 bp apart.

Results

Prior to clinical application, the protocol was tested on single buccal cells obtained from the heterozygous female patient and her unaffected partner. A total of 125 heterozygous single cells were analysed, 82% of which showed DNA amplification at both loci. Amplification failure at both loci was observed in 9% of the cells tested. In the remaining 9% amplification failure affected only one of the two loci, 4% at the mutation locus and 5% at the polymorphism locus. Of the cells which amplified successfully, 13% showed ADO at the mutation locus and 15% at the polymorphism locus.

The protocol was also tested on 15 blastomeres disaggregated from two spare day 3 embryos (one 7-cell and one 8-cell) donated for research with the written consent of the parents. Ten oocyte–cumulus complexes were retrieved from the patient following hyperstimulation, nine of which fertilized normally, as indicated by the formation of two pro-nuclei after ICSI. On day 3 of development, four embryos had reached the 8-cell stage, while the remaining five embryos had only attained the 4-cell stage. One cell each was biopsied from two of the 4-cell embryos, and these cells were biopsied and tested in order to obtain more data on the accuracy and reliability of the diagnosis. Two cells were biopsied from the remaining three 4-cell embryos and from two of the 8-cell embryos. The remaining two 8-cell embryos had three cells biopsied; in one case two cells that were tightly bound came out together, and in the other case a cell lysed post-biopsy and so a third cell was removed (Table I). Following multiplex nested PCR, the FGFR2 mutation fragment was amplified in 12/16 blastomeres and the D21S11 fragment in 13/16 blastomeres. After SSCP analysis of the mutation locus, five embryos were diagnosed as affected due to the presence of the mutant allele conformation in at least one cell. The remaining four embryos displayed only the normal allele conformation.

Results from two cells were available from two of the four embryos; of the other two embryos one had two cells available for analysis, but amplification failure occurred in one cell, and only one cell was biopsied from the fourth embryo. Thus, the latter two embryos were not considered for transfer. Representative results are shown in Figure 1. Analysis of the D21S11 locus in the two unaffected embryos with results from two cells displayed expected genotypes, and the two embryos were thus diagnosed as normal and transferred to the mother on day 4 post-fertilization, but no pregnancy resulted. The accuracy of the genetic diagnosis was assessed by analysing all the remaining cells from the embryos that were not transferred. The results obtained were concordant with those obtained following the diagnosis; five untransferred embryos were heterozygous carrying the mutant allele and the other two untransferred embryos were normal.

Second cycle of treatment

Eighteen oocyte–cumulus complexes were retrieved from the patient following hyperstimulation, 14 of which were successfully fertilized following ICSI. On day 3 post-fertilization, eight embryos consisted of less than six cells and six embryos contained six cells or more (Table II). Two blastomeres were biopsied from the embryos containing six cells or more (6/14). The embryos with less than six cells had one cell biopsied (8/14); these cells were biopsied and tested in order to obtain more data on the accuracy and reliability of the diagnosis.

Of the six embryos from which two cells were biopsied, it was possible to diagnose five. Successful DNA amplification of the FGFR2 fragment and D21S11 locus was achieved from both cells in all five embryos. Four embryos displayed the normal conformation upon SSCP analysis and displayed only the expected parental genotypes at the D21S11 locus. The fifth embryo clearly displayed the mutant conformation following SSCP analysis. By law in the UK a maximum of three embryos...
**Table I.** Summary of the results from the first cycle of treatment

<table>
<thead>
<tr>
<th>Embryo no.</th>
<th>Cells before biopsy (embryo grade)</th>
<th>Cells biopsied</th>
<th>Result mutation</th>
<th>Result STR (Mat/Pat)</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4 (1–)</td>
<td>1</td>
<td>N/M</td>
<td>221/246</td>
<td>Affected</td>
</tr>
<tr>
<td>2</td>
<td>8 (1–)</td>
<td>3*</td>
<td>N/N</td>
<td>226/246</td>
<td>Normal (ET)</td>
</tr>
<tr>
<td>3</td>
<td>4 (1–)</td>
<td>1</td>
<td>N/N</td>
<td>226/236</td>
<td>Normal</td>
</tr>
<tr>
<td>4</td>
<td>4 (1–)</td>
<td>2*</td>
<td>N/M</td>
<td>226/236</td>
<td>Affected</td>
</tr>
<tr>
<td>5</td>
<td>8 (2+)</td>
<td>3**</td>
<td>N/N</td>
<td>221/246</td>
<td>Normal (ET)</td>
</tr>
<tr>
<td>6</td>
<td>8 (1–/2+)</td>
<td>2</td>
<td>N/M</td>
<td>221/236</td>
<td>Affected</td>
</tr>
<tr>
<td>7</td>
<td>7 (1–)</td>
<td>2</td>
<td>N/N</td>
<td>226/236</td>
<td>Normal</td>
</tr>
<tr>
<td>8</td>
<td>5 (1–)</td>
<td>2</td>
<td>N/M</td>
<td>221/236</td>
<td>Affected</td>
</tr>
<tr>
<td>9</td>
<td>4 (1–)</td>
<td>2</td>
<td>N/M</td>
<td>226/236 M*</td>
<td>Affected</td>
</tr>
</tbody>
</table>

*One cell lost during biopsy.

**Two cells removed together.

FGFR2 locus: diagnosed either as normal (N) or affected (M).


Allele drop-out denoted by (*)

Amplification failure at both loci denoted by (–)

Normal (ET) = embryos transferred.

Embryo grade as determined by embryologist prior to biopsy and re-evaluated on day 4 prior to embryo transfer.

*Figure 1.** Results of the first clinical cycle. Single-stranded conformation polymorphism (SSCP) of the FGFR2 fragment encompassing the G/A substitution at codon 568. Lanes 1 and 9: maternal buccal cells as a heterozygous affected control. Lanes 2 and 7: normal blastomeres (embryos 3 and 7 respectively). Lanes 3–5: three heterozygous affected blastomeres (embryos 4, 6 and 8 respectively). Lane 6: faint amplification from which a result could not be obtained (cell 2, embryo 8). Lane 8: homozygous affected blastomere due to ADO of the normal allele (cell 2, embryo 9).

Preimplantation genetic diagnosis for Crouzon syndrome can be transferred in any one IVF cycle, and therefore the three embryos with the best morphology were selected for transfer. Two of the embryos which were selected had consisted of eight cells prior to biopsy and the third was a 9-cell embryo. The remaining normal embryo which was not transferred had consisted of six cells prior to biopsy and was then reduced to four cells. The transfer resulted in an ongoing pregnancy which at the time of submission of this paper was in week 31. The patient has refused prenatal testing; however, ultrasound examinations, which are capable of detecting craniosynostosis in vivo (Gollin et al., 1993), have not revealed any abnormalities. However, due to the phenotypic variability observed in Crouzon syndrome, a definitive diagnosis would require molecular profiling.

Lower amplification efficiencies were obtained from the developmentally delayed embryos. One cell each was biopsied from eight embryos. Of these, amplification of both fragments was observed in three out of eight cells. Each of the three cells displayed the mutant conformation following SSCP analysis. The results of the genetic diagnosis were confirmed by the results obtained from the embryos that were not transferred. Additionally, it was possible to determine that four of the undiagnosed embryos were carrying the mutation and the other two were normal.

**Discussion**

Crouzon syndrome is characterized by cranial synostosis, exophthalmos and midface hypoplasia. Craniosynostosis is not
lethal, but treatment requires surgical intervention from a young age to alleviate pressure build-up on the cranial vault. It was following the death of the couple’s only child aged 18 months from complication of such surgery, that they opted for PGD. Crouzon syndrome is a good candidate for PGD as the disorder is not lethal but treatment does entail certain risks. Therefore, whilst certain couples such as the patients in question would not consider termination of pregnancy following a positive prenatal diagnosis, PGD did offer them a viable alternative.

The single-cell PCR assay described in this paper has shown a satisfactory level of amplification efficiency considering the products of the FGFR2 locus underwent nested amplification and subsequent detection by silver staining. Results may have been obtained from a larger number of cells had it been possible to detect the products using a more sensitive automated laser fluorescence. However, this approach was not possible as the mutant allele did not produce a novel conformation upon SSCP analysis under a wide range of conditions when analysed on the ALFexpress®. The accuracy of the test was evaluated by analysing all un-transferred embryos. In each case the results were concordant with those obtained during the diagnosis. However, due to the potentially high risk of misdiagnosis, calculated at 13% following the analysis of a single blastomere, it was decided to only transfer embryos from which results were available from two cells. This strategy reduces the risk of misdiagnosis due to ADO whilst circumventing the need to test two cells.

At the time this assay was developed, two intragenic single nucleotide polymorphisms had been described in the gene (Moloney et al., 1996). However, the couple were found to be uninformative at both. The genomic structure of the gene has since been elucidated (Zhang et al., 1999) and sequenced to detect more polymorphisms (Glaser et al., 2000). The latter study revealed a further two intragenic polymorphisms which could also be considered for use in future cases.

In conclusion, we have developed a single cell assay capable of detecting Crouzon syndrome caused by a G→A transition at codon 338 in FGFR2 using multiplex nested PCR and silver-stained SSCP analysis. A highly polymorphic STR, D21S11, located on chromosome 21, was simultaneously amplified and analysed as a control against some forms of contamination.

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Note added in proof
Since this article was submitted for review, the pregnancy has developed to term and the patient has given birth to a healthy girl weighing 2.94 kg. A sample of fetal blood was collected from the umbilical cord during the delivery procedure and used to confirm...
the results of the PGD. The tests confirmed that the baby was not carrying the causative mutation. This paper therefore describes the first live birth following PGD for Crouzon syndrome.

References


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