

Article

Birth of a healthy female after preimplantation genetic diagnosis for Charcot–Marie–Tooth type X



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Abstract

The X-linked dominant form of Charcot–Marie–Tooth syndrome (CMTX) is a clinically and genetically heterogeneous hereditary disorder of the peripheral nerves caused by mutations in the *GJB1* gene that encodes a gap junction protein named connexin 32 (Cx32). Clinically, CMTX is characterized by peripheral motor and sensory deficit with muscle atrophy. A couple with a previous history of pregnancy termination after being diagnosed positive for CMTX by chorionic villus sampling, was referred for preimplantation genetic diagnosis (PGD). The female partner carried the causative H94Q, characterized by a C→G substitution in codon 94 of exon 2 of the *GJB1* gene. Embryos obtained after intracytoplasmic sperm injection (ICSI) were evaluated for the presence of the mother's mutation using polymerase chain reaction (PCR), followed by mutation analysis performed using the minisequencing method. Amelogenin sequences on the X and Y chromosomes were also co-amplified to provide a correlation between embryo gender and mutation presence. A single PGD cycle was performed, involving nine fertilized oocytes, five of which developed into good quality embryos useful for biopsy. Two unaffected embryos were transferred, resulting in a singleton pregnancy followed by the birth of a healthy female.

Keywords: Charcot–Marie–Tooth, minisequencing, preimplantation genetic diagnosis, single cell PCR

Introduction

Charcot–Marie–Tooth (CMT) disease is the most common genetic disorder of the peripheral nervous system, with an overall prevalence estimated to be approximately 1:2500. The typical clinical symptoms involve distal muscle weakness and atrophy, often associated with mild to moderate sensory loss, depressed tendon reflexes, and high-arched feet.

CMT type X (CMTX) represents an X-linked form of CMT; it is inherited in a dominant manner and accounts for 10–20% of patients with the CMT syndrome. Males with CMTX have a progressive peripheral motor and sensory neuropathy that tends to be more severe than that seen in other forms of CMT. Females may be normal or have mild to moderate symptoms.

CMTX disease is caused by mutations in the gene for connexin 32 (Cx32; also known as gap junction gene b1; GJB1), a member of the family of gap junction proteins (Bergoffen et al. 1993). The *GJB1* gene spans about 9 kb and exon 2 contains the entire open reading frame (ORF) of approximately 850 bp. About 90% of Cx32 mutations are located in exon 2.

The detection of an affected pregnancy and its subsequent termination is critical, because it allows a couple to consider pregnancy interruption as an option. Preimplantation genetic diagnosis (PGD) represents a possible alternative to prenatal diagnosis in these cases, avoiding the risk of pregnancy termination because unaffected embryos are pre-selected before the pregnancy is established (Verlinsky et al., 1990; Handyside et al., 1989; Kuliev and Verlinsky, 2002; Sermon, 2002).

This report describes a protocol optimized for the detection of a CMTX causing mutation at the single cell level. The presented method involves DNA amplification using a multiplex nested polymerase chain reaction (PCR), for the simultaneous amplification of a fragment of exon 2 of the *GJB1* gene encompassing the mutation as well as of sequences located on the X amelogenin gene and its Y pseudogene for sex determination (Nakahori *et al.*, 1991). The obvious advantage of this co-amplification is to provide a correlation between embryo gender and mutation presence, identifying and subsequently transferring healthy female as well as non-affected male embryos from the cohort of embryos under investigation. The minisequencing method (Fiorentino *et al.*, 2003) was then used to detect the mutation of interest. This procedure was used for PGD of CMTX disease resulting in the birth of a healthy female child.

Materials and methods

A young non-consanguineous couple (woman 28 years old, man 31 years old), with no history of infertility, was referred after having one pregnancy terminated following the diagnosis of a CMTX affected male fetus. The patient had neuropathy, with abnormal signs on muscle myography and mild clinical symptoms. Severe pathology was present in the patient's brother and uncle.

Genetic diagnosis, performed by a different centre, indicated that the female partner was a carrier of the H94Q mutation of the *GJB1* gene. This mutation involves a C→G single base pair substitution at codon 94, located in exon 2 of the *GJB1* gene, that determines a His→Gln amino acid substitution.

To confirm the genetic status of the female partner of the couple, PCR amplification of a fragment of exon 2 of the *GJB1* gene encompassing the mutation of interest was performed, using outer oligonucleotide primers listed in **Table 1**. Mutation analysis was carried out by direct sequencing of the obtained PCR product. For mutation detection, a minisequencing primer (**Table 1**) was designed for the H94Q mutation and initially tested on sequenced PCR products.

In order to evaluate the amplification efficiency on single cells and allelic drop-out (ADO) rate, primers used for the detection

of the above mutation were preliminarily tested on single lymphocytes, together with amelogenin primers for sex determination.

The PGD cycle was performed using a standard IVF protocol described elsewhere (Rienzi *et al.*, 1998). Embryo biopsy was carried out removing two blastomeres from embryos that contained >6 cells and <50% fragmentation, while only one cell was taken from embryos with ≤6 cells and <50% fragmentation (Delhanty and Handyside, 1995; Gianaroli *et al.*, 1997; Verlinsky and Kuliev, 2000). After removal, each blastomere biopsied was transferred into sterile 0.2 ml PCR tubes containing 5 µl of alkaline lysis buffer (200 mmol/l KOH, 50 mmol/l dithiothreitol), covered with mineral oil (Sigma-Aldrich, Milan, Italy) and finally subjected to cell lysis, incubating them at 65°C for 10 min. The alkaline lysis buffer was then neutralized by adding 5 µl of neutralization buffer (900 mmol/l Tris-HCl, 300 mmol/l KCl, 200 mmol/l HCl) before processing for PCR.

The PCR strategy comprised an initial multiplex external PCR for the simultaneous amplification of a fragment of exon 2 of the *GJB1* gene encompassing the mutation H94Q and amelogenin gene for sex determination. Two separate PCR reactions were then performed, a nested PCR for amplification of the specific *GJB1* gene region and a semi-nested PCR for amelogenin gene amplification.

For sex determination, dye labelled PCR products were generated using Amelogenin Inner Forward primer coupled with 6-FAM dye (Applied Biosystems, Foster City, CA, USA), in conjunction with unlabelled Outer/Inner Reverse primer (**Table 1**).

Mutation analysis for CMTX was carried out using a minisequencing method, as described elsewhere (Fiorentino *et al.*, 2003). Subsequently, the remaining embryos that were not transferred were collected in individual tubes and reanalysed to verify the PGD results. During pregnancy, the genetic status of the fetus was confirmed by an independent laboratory on DNA extracted from chorionic villus tissue. Additionally, DNA extracted from cord blood of the newborn at birth was analysed.

Table 1. Description of primers used in PCR amplification and minisequencing reaction. T^{ann}. = annealing temperature, 6-FAM = fluorescent dye.

| Name | Primer sequence (5' to 3') | T ^{ann} (°C) | Size (bp) |
|------------------------------|--------------------------------------|-----------------------|-----------|
| GJB1 | | | |
| Outer forward | 5'-CTATGACCAATTCTTCCCCATCTC-3' | 55 | 262 |
| Outer reverse | 5'-ATACATGAAGACGGCCTCAAACA-3' | | |
| Inner forward | 5'-GCAGCTCATCCTAGTTTCCACC-3' | | 181 |
| Inner reverse | 5'-CACGCTGATGACATAGGTCCAC-3' | | |
| Amelogenin | | | |
| Outer forward | 5'-CACCCCTGGTTATATCAACTTCAGC-3' | 55 | |
| Outer/inner reverse | 5'-ATCAGAGCTTAAACTGGGAAGCTG-3' | 55 | 103/109 |
| Inner forward | 5'-6-FAM-CCCTGGGCTCTGTAAAGAATAGTG-3' | | |
| Minisequencing primer | 5'-TTTTCTATGTGTGCTGGTGAGCCAC-3' | 50 | 26 |

Results and discussion

Preliminary experiments on single lymphocytes collected from the normal male partner (25 lymphocytes) and from the female carrier (50 lymphocytes) of the couple have shown a high amplification efficiency and an acceptable ADO rate. A summary of the results is shown in **Table 2**. During the set-up of this PGD assay, one polymorphic linked STR marker (DXS106) was investigated for ADO evaluation, but the results were not informative for the couple under study (data not shown). Thus, the assumption that the ADO rate observed in the lymphocytes tested is approximately the same as in the biopsied blastomeres during PGD implies that in 8.3% of the embryos, ADO could occur. In male embryos, due to the presence of only one X chromosome, this eventuality will determine an amplification failure and would not result in the transfer of an affected embryo, but would only reduce the number of unaffected embryos transferable. In female embryos, instead, the probability of an ADO occurrence of the mutant allele can be estimated to be about 2%. However, by analysing two blastomeres from the same embryo, the chance of misdiagnosis of a female carrier embryo will be reduced to 0.2%, i.e. 0.6% of the probability of an ADO occurrence in both cells (0.083^2), of the mutant allele (0.5) of a female embryo (0.5). Therefore, with the optimized system sufficiently robust and accurate, PGD analysis was performed even without the use of linked STR markers, selecting for transfer only embryos for which results from two blastomeres were available.

A single PGD cycle was performed, with 12 oocytes collected, yielding nine zygotes with two pronuclei after ICSI. On day 3 of development, in accordance with the embryo score system (Rienzi *et al.* 2002), five embryos were classified as good

Table 2. PCR amplification efficiency and allele drop-out (ADO) rate from single lymphocytes. NE = not evaluated.

| Gene | PCR amplification efficiency (%) | ADO (%) |
|--------------|----------------------------------|------------|
| GJB1 | 70/75 (93.3) | 4/48(8.3) |
| Male cells | 22/25 (88.0) | NE |
| Female cells | 48/50 (96.0) | 4/48(8.3) |
| Amelogenin | 72/75 (96.0) | 2/23 (8.7) |
| Male cells | 23/25 (92.0) | 2/23 (8.7) |
| Female cells | 49/50 (98.0) | NE |

Table 3. Summary of the results from the cycle of treatment. GJB1 locus: diagnosed either as normal (N) or mutated (M). Amplification failure at both loci denoted by (-). Allele drop-out (ADO) denoted by (*) AE = arrested embryos, ET = embryos transferred.

| Embryo no. | Cells before biopsy | Cells biopsied | Mutation analysis results | Sex diagnosis results | Stage of division at day 5 | Diagnosis |
|------------|---------------------|----------------|---------------------------|-----------------------|----------------------------|---------------------------|
| 1 | 7 | 2 | N, N | X Y, X Y | Pre-blastocyst | Normal male (ET) |
| 2 | 8 | 2 | N/M, -/M* | X X, X X | 7 cells (AE) | Carrier female |
| 3 | 7 | 2 | M, M | X Y, -Y* | Pre-blastocyst | Affected male |
| 4 | 8 | 2 | N/N, N/N | X X, X X | Pre-blastocyst | Normal female (ET) |
| 5 | 5 | 1 | - | - | 12 cells | No result (cryopreserved) |

quality and selected for embryo biopsy. Four embryos (1, 2, 3 and 4) had reached the 7–8 cell stage, while one embryo (5) had only five cells. The remaining four fertilized oocytes developed into poor quality embryos.

After PCR amplification of both *GJB1* locus and *amelogenin* gene, followed by minisequencing mutation analysis, an amplification rate of 86.7% (8/9 blastomeres) was obtained for both loci (**Table 3**). One embryo (no. 3) was diagnosed as an affected male, due to the presence of the mutant allele in homozygosis, and the X and Y chromosome amplification from the amelogenin locus. One embryo (no. 2) displayed one normal allele and one mutant allele, showing only amplification of the X chromosome, and was then diagnosed as a heterozygous female. The remaining two embryos displayed only the normal allele, one of which (no. 4) showed amplification of the X chromosome, and was diagnosed as a normal female. The other (no. 1) showed amplification of the X and Y chromosomes, and was diagnosed as a normal male. No results were obtained from the fifth embryo (no. 5), from which only one blastomere was biopsied, due to PCR failure. Furthermore, one male embryo (no. 3) did not amplify the X-*amelogenin* sequence while amplifying the Y-*amelogenin*, revealing an ADO occurrence (1/9; 11.1%). One female embryo (no. 2) showed another occurrence of ADO in the *GJB1* locus, lacking the mutant allele in one of the two blastomeres biopsied (1/9; 11.1%). No contamination was detected in blank controls collected during the biopsy procedure, or in the blanks from the PCR reagents.

The results for the investigated blastomeres in the present PGD case are illustrated in **Figure 1**. For the minisequencing technique, colour was assigned to individual ddNTPs as follows: green/A, black/C, blue/G, red/T. The minisequencing reaction produces one (homozygote) or two (heterozygote) peaks depending on the genotype at this locus. One of the two peaks observed in heterozygous blastomeres comes from the normal base, the other from the mutated base. The size of each peak is determined by the length of the primer, as well as by the specific dye-labelled ddNTP incorporated, the chemical structure of which produces different electrophoretic mobility. This could result in the visualization of two separate peaks or two superimposed peaks of different colours in heterozygous samples (Fiorentino *et al.*, 2003).

Panels A–E, B–F, C–G and D–H in **Figure 1** represent minisequencing results obtained from embryos 1, 2, 3 and 4 respectively. In panels A, D, E and H, the minisequencing

window in the upper panel shows the presence of only one blue peak (normal allele, wild type base G). Panel B, instead, shows the presence of two different-coloured peaks: one refers to the normal allele (blue peak) and the other to the mutated allele (black peak, mutant base C). Panels C and G display the presence of only one black peak (mutated allele). Moreover, panel F illustrates the result obtained from the second cell biopsied from embryo no. 2 that displays an ADO occurrence in Cx32 locus, deduced from the presence of mutated allele only (black peak, mutant base C).

For amplification of the amelogenin X and Y sequences, a region of high homology between the X amelogenin and its Y

pseudogene was selected (Nakahori *et al.*, 1991). Primers were designed at a location where there was 100% sequence homology between the X and Y amelogenin sequences. These primers (**Table 1**) flank a 6 bp deletion within intron 1 of the X homologue, resulting in 104 bp and 110 bp PCR products from X and Y chromosomes respectively. This size difference makes it possible to perform sex identification and is sufficient to allow easy discrimination between the two peaks (X and Y) after capillary electrophoresis. Representative results are shown on the bottom of panels A–E, B–F, C–G and D–H for embryos 1, 2, 3 and 4 respectively. Panel G displays an ADO occurrence for amelogenin locus, deduced from the presence of the Y-chromosome peak only obtained for the second cell

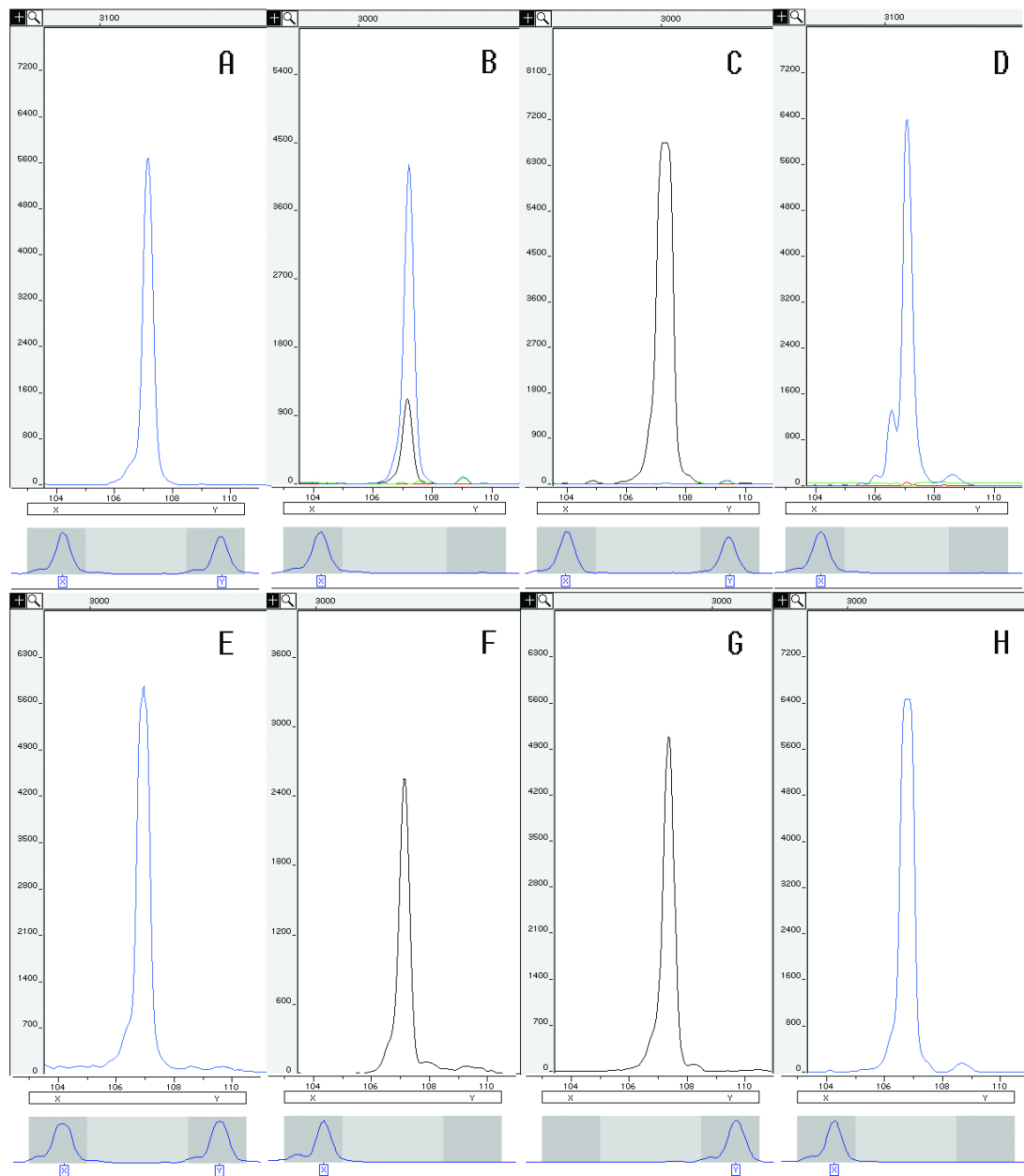


Figure 1. Results for blastomeres investigated in the present PGD case. (A–E), (B–F), (C–G) and (D–H) illustrate minisequencing results (top) and sex testing results (bottom) obtained from the cells (two blastomeres per embryo) biopsied from embryos 1, 2, 3 and 4 respectively (see explanation in the text).

biopsied from embryo no. 3.

Embryos that were not transferred (one affected, no. 3; and one heterozygotes, no. 2) were reanalysed to confirm the accuracy of the genetic diagnosis. The results were concordant with those obtained following by previous PGD, and none of the tested embryos gave discordant results.

Only healthy embryos (nos. 1 and 4) were transferred; the transfer resulted in a singleton pregnancy, confirmed as a healthy female by chorionic villus sampling at 12 weeks, in agreement with the PGD result and by analysis of cord blood lymphocytes of the newborn.

Although CMTX is not a fatal disease and the disorder does not affect normal life expectancy, its relatively high frequency and its clinical outcome make it a good candidate for the development of PGD cycles. This could avoid couples with a family history of the disease having to consider pregnancy termination following a positive prenatal diagnosis. The single cell assay developed here for CMTX may give hope to couples who find themselves in a similar situation.

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Received 17 June 2003; refereed 16 July 2003; accepted 10 September 2003.