Pregnancy after preimplantation genetic diagnosis for Charcot–Marie–Tooth disease type 1A

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Charcot–Marie–Tooth (CMT) disease type 1A is an autosomal dominant peripheral neuropathy characterized by slow progressive distal muscle wasting and weakness, and decreased nerve conduction velocities. Most CMT1A cases (>98%) are caused by a duplication of a 1.5 Mb region on the short arm of chromosome 17 containing the PMP22 gene. A couple with a previous history of CMT followed by termination of pregnancy was referred to our centre for preimplantation genetic diagnosis (PGD). The husband carries the CMT1A duplication which can be detected by polymerase chain reaction (PCR) analysis using polymorphic (CA)n markers localized within the duplication. PCR amplification of genomic DNA of the parents-to-be with one of the two primers labelled with fluorescein, followed by automated laser fluorescence (ALF) gel electrophoresis of the amplified fragments allows the distinction between both genotypes. Embryos obtained after intracytoplasmic sperm injection (ICSI) were evaluated for the presence of the normal allele of the father. PCR with single Epstein–Barr virus-transformed lymphoblasts and blastomeres resulted in 91.4 and 93.5% amplification efficiency respectively, whereas none of the blank controls gave a positive signal. Allele drop-out (ADO) was observed in eight out of 32 lymphoblasts (25%) or in five out of 21 blastomeres (23.8%). However, within this set-up ADO will never lead to transfer of an affected embryo. A first ICSI–PGD cycle did not result in embryo transfer for the patient. A second cycle involved 10 mature oocytes of which eight were fertilized, resulting in five embryos for biopsy. Two unaffected embryos were available for transfer and resulted in a singleton pregnancy. The genotype of the fetus has been confirmed healthy by chorionic villus sampling.

Key words: allele drop-out/Charcot–Marie–Tooth disease/ICSI/preimplantation genetic diagnosis

Introduction

Charcot–Marie–Tooth (CMT) disease is the commonest inherited peripheral neuropathy in humans, with a prevalence rate of 1/2500 (Skre et al., 1974; Patel and Lupski, 1994). The most prevalent form of the disease, CMT type 1A or hereditary motor and sensory neuropathy type I is characterized clinically by distal muscle atrophy and weakness, and electrophysiologically by decreased nerve conduction velocities. It is an autosomal dominant disease characterized by motor and sensory impairment in a variable degree and is usually progressive. Some patients may almost be asymptomatic while others may lose their ability to walk. Most patients are disabled to some degree. Lower limbs are generally more severely affected than upper limbs. Clinical onset usually occurs in the second or third decade of life (average age of onset 12.2 ± 7.3 years; Bird and Kraft, 1978). In a majority of cases (≥70%), the genetic basis of the disease involves a 1.5 megabase chromosomal duplication on the short arm of chromosome 17 (17p11.2) that includes the entire PMP22 gene (Nelis et al., 1996). PMP22 is expressed by Schwann cells and is localized mainly in compact peripheral nervous system myelin (Suter and Snipes, 1995). A gene dosage effect resulting in mild overexpression of the protein (theoretically 1.5-fold) has been suggested as the mechanism underlying the demyelinating neuropathy in CMT1A (Warner et al., 1996).

Molecular diagnosis of CMT1A requires detection of the duplication. This can be done using different molecular genetic methods that, essentially, make use of DNA probes located in the duplicated region (Blair et al., 1995; Navon et al., 1995). Prenatal diagnosis for CMT1A is feasible using these molecular genetic methods (Navon et al., 1995). Multicolour fluorescence in-situ hybridization (FISH) has also been used for prenatal diagnosis for CMT1A (Lebo et al., 1993). Detection of an affected fetus with the option to terminate the pregnancy is very difficult to cope with for the parents-to-be. A possible alternative in these cases is preimplantation genetic diagnosis (PGD) (Liebaers et al., 1992; Handyside, 1993; Winston and Handyside, 1993). This very early form of prenatal diagnosis performed on embryos obtained through in-vitro fertilization (IVF) offers couples at risk the possibility of selecting unaffected embryos for transfer and thus prevention of termination of pregnancy. Embryos are obtained via IVF with intracyto-
plasmic sperm injection (ICSI) and their disease status is determined by DNA analysis of one or two blastomeres biopsied from each embryo.

The present report describes a polymerase chain reaction (PCR)-based assay that was developed for the detection of the CMT1A mutation and its use for PGD in a couple where the husband carries the 1.5 Mb duplication on chromosome 17 and suffers from CMT1A. Using this assay, two unaffected embryos were selected for transfer during the second treatment cycle. This resulted in an unaffected ongoing singleton pregnancy.

Materials and methods

Collection of single cells

Preliminary PCR experiments were performed on both Epstein–Barr virus (EBV)-transformed lymphoblasts and blastomeres derived from ICSI embryos that were unsuitable for transfer or cryopreservation and therefore used for research. Agreement was obtained from the donating patients and the institutional ethical committee. Single cell PCR was also performed on spermatozoa from the husband.

EBV-transformed lymphoblasts were cultured according to standard procedures (Ventura et al., 1988) and collected in 2.5 μl alkaline lysis buffer (ALB, 200 mM KOH, 50 mM dithiothreitol) as described (Sermon et al., 1998). The collection of research embryo blastomeres has also been described (Sermon et al., 1998).

The husband’s semen was obtained by masturbation into a sterile container. After liquefaction for ~30 min at 37°C, the semen parameters were assessed by established procedures (Kruger et al., 1986; World Health Organization, 1992). Fresh or frozen sperm samples were further processed using a Percoll gradient centrifugation as described previously (De Vos et al., 1997). Single motile sperm cells were selected, immobilized and washed three times in separate washing droplets of HEPES-buffered Earle’s medium with 10% w/v polyvinylpyrrolidone before transfer to 200 μl PCR tubes containing 2.5 μl ALB.

All samples were kept at –80°C for no longer than 1 week until further processing.

ICSI procedure

Ovarian stimulation was done as previously described (Ubaldi et al., 1995). The combination of vaginal ultrasound results and serum oestradiol concentrations indicated that at least nine follicles were present (Vandervorst et al., 1998). Oocyte retrieval was done by vaginal ultrasound-guided puncture of the ovarian follicles, 36 h after human chorionic gonadotrophin administration. Removal of surrounding cumulus and corona cells included an enzymatic hyaluronidase step followed by mechanical denudation (Van de Velde et al., 1997). The ICSI procedure on mature metaphase-II oocytes was performed as described by Van Steirteghem et al. (1995).

Fertilization was examined ~16–18 h after ICSI. After another 24 h in-vitro culture, the cleavage characteristics of the fertilized oocytes were evaluated: numbers and sizes of blastomeres and the presence of anucleate cytoplasmic fragments were recorded (Nagy et al., 1995). Further development was evaluated on day 3 after injection, just prior to embryo biopsy.

Embryo biopsy

Embryo biopsy was carried out in the morning of day 3 after oocyte microinjection. Acidic Tyrode’s solution was used to drill a hole in the zona pellucida. Depending on the cell stage, one blastomere (if the embryo had less than seven cells) or two blastomeres (if the embryo had seven cells or more) containing a clear nucleus were gently aspirated through the hole. When embryo compaction or sticky blastomeres complicated the biopsy procedure, embryos were incubated in calcium- and magnesium-free medium (136.8 mM NaCl, 2.7 mM KCl, 0.4 mM NaH₂PO₄, 5.6 mM glucose, 3.4 mM EDTA, 11.9 mM NaHCO₃, 0.01% w/v phenol red) (supplemented with 0.5% human serum albumin) for ~5–10 min prior to biopsy. After biopsy, the blastomeres were washed three times in calcium- and magnesium-free medium (containing 0.4% bovine serum albumin) and transferred to a 200 μl PCR tube containing 2.5 μl ALB as described for research blastomeres. The tubes were kept at –80°C for at least 30 min.

Cell lysis and PCR procedure

The cells were lysed by incubating them at 65°C for 10 min. The ALB was then neutralized with 2.5 μl of neutralization buffer (NB; 900 mM Tris–HCl pH 8.3, 300 mM KCl, 200 mM HCl). The reaction mix for the fluorescent PCR was contaminated with the restriction enzyme NalIII by incubation at 37°C for at least 3 h, followed by inactivation of the enzyme by incubation at 65°C for at least 30 min. The following primer set was used: AFM191×h12a 5’-CTTGGAC-TTCTACAAATCCTGGCA and AFM191×h12m 5’-GGCACCATAATCAGTGTGAACACAT, located on locus D17S921 (Gyapay et al., 1994). The primer AFM191×h12a was labelled with a fluorescein (Eurogentec, Seraing, Belgium), while the primer AFM191×h12m remained unlabelled. Reaction mix was added to the cells to a final volume of 25 μl and final concentrations of 50 mM KCl, 10 mM Tris–HCl pH 8.3, 2 mM MgCl₂, 0.1 mg/ml gelatin, 0.2 mM dNTP, 0.2 μM primers and 1.25 units Taq polymerase (Perkin Elmer, Brussels, Belgium). The PCR was carried out on a Perkin Elmer Cetus GeneAmp PCR System 2400 using the following programme: 5 min denaturation at 96°C, followed by 10 cycles of 30 s at 96°C, 30 s at 58°C and 30 s at 72°C, followed by 35 cycles of 30 s at 94°C, 30 s at 58°C and 30 s at 72°C, followed by 6 min at 72°C. Three μl of the PCR product was mixed with 3 μl loading buffer (0.0125 g bromophenol blue, 2 ml glycerol, 3 ml H₂O) and loaded on a 6% denaturing polyacrylamide gel [bis-acrylamide ratio 19:1, 7 M urea, 1× 1.0 M Tris, 0.9 M Boric acid, 0.01 M EDTA (TBE)]. The electrophoresis unit used was an ALF Automated DNA Sequencer from Pharmacia Biotech. The results were processed using the Fragment Analyser software provided by the manufacturer.

Patient description

The couple was referred to our centre after 3 years of unexplained primary infertility followed by a spontaneous pregnancy which resulted in a spontaneous abortion. Further infertility treatment of the couple in a peripheral centre involved four failed intrauterine insemination cycles in which IVF was attempted, which resulted in a twin pregnancy. Prenatal diagnosis for CMT1A was considered because of the recent CMT1A diagnosis in the male partner. Peripheral nerve electrophysiological investigations for a cervical disc herniation revealed significantly reduced motor and sensory nerve conduction velocities suggesting a peripheral motor and sensory neuropathy. Clinical and neurological examination showed absence of the ankle tendon reflexes and atrophy of the feet muscles. The diagnosis of a hereditary motor and sensory neuropathy type 1 was proven by the presence of the CMT1A duplication in the asymptomatic proband. The DNA analysis was done using Southern blot analysis of markers recognizing restriction fragment length polymorphisms (Raeymeekers et al., 1992) and CA repeat polymorphisms (Navon et al., 1995) located in the CMT1A duplication. The prenatal diagnosis revealed two affected fetuses and the pregnancy was therefore interrupted. In
order to avoid another termination of pregnancy, the couple opted for PGD.

Results

The patient carries the CMT1A duplication, which can be detected by PCR analysis using polymorphic (CA)ₙ markers localized within the duplication. The couple is informative for the (Aₙ) repeat AFM191xh12. PCR amplification of genomic DNA of the parents-to-be with the primer set AFM191xh12a/AFM191xh12m, where the former primer was labelled with fluorescein, followed by automated laser fluorescence (ALF) gel electrophoresis of the amplified fragments allowed the distinction between both genotypes. The patient has genotype 11.2, with a duplication of allele 1, whereas the female partner has genotype 1.3 for this marker. The genotype of the duplicated AFM191 marker was determined by segregation analysis in the patient’s family. In Figure 1, lanes 7 and 8 represent the ALF gel electrophoresis profiles of the amplified genomic DNA of the parents-to-be, showing the separation of the three different alleles. Both profiles display a peak at 183 bp, representing allele 1. For the female partner (lane 7), a second peak at 175 bp corresponds to allele 3, whereas the patient (lane 8) has a second peak at 179 bp, representing allele 2. Indicative for the duplication of allele 1 in the patient is that the largest PCR fragment peak (183 bp, allele 1) is somewhat higher than the peak representing the smaller PCR fragment (179 bp, allele 2). For PGD, the presence of the healthy allele 2 of the patient was evaluated.

The efficiency and accuracy of the PCR was evaluated in preliminary experiments on single EBV-transformed lymphoblasts with the known genotype of the parents-to-be and on research embryo blastomeres (Table I). PCR on single lymphoblasts and blastomeres resulted in 91.4 and 93.5% amplification efficiency respectively, whereas none of the blank controls gave a positive signal. ADO was observed in eight out of 32 lymphoblasts (25%) or in five out of 21 blastomeres (23.8%). Eight blastomeres (resulting from three embryos) were homozygous for the tested markers and could not be included for the ADO rate estimation. The ADO rate is rather high, but within this set-up ADO will never lead to transfer of an affected embryo.

Genomic DNA of the prenatal diagnosis of the twin pregnancy was obtained and the genotypes of the two fetuses were confirmed using our approach. Both had inherited the duplication of the father: one had genotype 11.3 and the other had genotype 11.1. Two supernumerary embryos cryopreserved within the IVF cycle leading to the above pregnancy were donated for research. PCR analysis of their genotype was considered as a final validation of our technique prior to clinical application in PGD. Although the analysis was successful, both embryos displayed the same affected genotype 11.3, having inherited the duplication of the father.

For PGD with PCR, absolute preference is given to ICSI, in order to prevent DNA contamination from sperm cells present in the zona pellucida. A first ICSI cycle involved seven mature oocytes and four germinal vesicle-stage oocytes, which were matured in vitro and were injected the next day.

Table I. Preliminary single cell polymerase chain reaction experiments with Epstein–Barr virus transformed lymphoblasts (n = 35) and blastomeres (n = 31) derived from 11 research embryos

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Lymphoblasts (%)</th>
<th>Blastomeres (%)</th>
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<tbody>
<tr>
<td>Amplification</td>
<td>91.4 (32/35)</td>
<td>93.5 (29/31)</td>
</tr>
<tr>
<td>Contamination</td>
<td>0.0 (0/35)</td>
<td>0.0 (0/31)</td>
</tr>
<tr>
<td>Allele drop-out</td>
<td>25.0 (8/32)</td>
<td>23.8 (5/21)</td>
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For blastomeres, the allele drop-out rate is expressed per number of heterozygous blastomeres (21 blastomeres corresponding to eight embryos were heterozygous, the other three embryos or eight blastomeres were homozygous).

Figure 1. Automated laser fluorescence gel electrophoresis of the first preimplantation genetic diagnosis (PGD) cycle. Lane numbers are indicated on the right side of the figure and the y-axis indicates the peak size in bp. Lane 9 shows a 50 bp ladder standard (150 and 200 bp fragments are present). Amplified genomic DNA of the parents-to-be was used as a control (lanes 7 and 8). Lane 7 is the amplified DNA of the female partner (genotype 1.3). Allele 1 resides at 183 bp, whereas the 175 bp peak represents allele 3. The amplified DNA of the patient (lane 8) shows the affected (duplicated) allele 1 at 183 bp and the healthy allele 2 at 179 bp. The presence of the latter allele is evaluated for PGD. Lane 10 represents the profile of an embryo with the affected genotype 11.3. Three such embryos were obtained. Lane 18 represents an embryo homozygous for allele 1. Four embryos were found to have this affected genotype. Lanes 1 and 2 represent the amplification of two blastomeres biopsied from the same embryo for which ‘no diagnosis’ was concluded.
In total, 10 oocytes were fertilized, and because they all cleaved well, 10 embryos were available for embryo biopsy. From three embryos, two nucleated blastomeres were available for diagnosis. A single blastomere was removed from the other seven embryos, five of which clearly revealed the presence of a nucleus. Amplified genomic DNA of both parents-to-be was used as a control (Figure 1, lanes 7 and 8). Seven embryos were shown to have inherited the duplicated allele 1 from the patient: three embryos displayed genotype 11.3 and four embryos were homozygous for allele 1. One example of each genotype is shown in Figure 1, lanes 10 and 18 respectively. Three embryos remained without diagnosis, two of which were derived from the in-vitro matured germinal vesicle stage oocytes. From these, only a single blastomere could be taken for diagnosis. In one blastomere no nucleus was observed and in the other the presence of a nucleus was uncertain, explaining the lack of amplification. The lack of diagnosis in the third embryo was due to unclear results in the two blastomeres that were biopsied, which did not give exactly the same profile (Figure 1, lanes 1 and 2). The peaks were rather small, slightly above the background. Whereas in lane 2 the presence of the healthy allele 2 of the father is suggested, its presence in lane 1 is uncertain. Based on these uncertain results, the risk of diagnosing this embryo as a healthy one was not taken. Overall, this resulted in no transfer for the patient within this PGD cycle.

All the embryos were used for confirmation of the diagnosis or for further analysis when no diagnosis had been obtained. For five out of the seven affected embryos, confirmation of the diagnosis was obtained in two or more nucleated blastomeres. For one embryo no nucleated blastomeres were available for confirmation and another one revealed an ADO of allele 2 during PGD. This ADO could not be noticed during PGD because only one blastomere was available for analysis. This resulted in the loss of a healthy embryo for transfer. Of the two embryos which failed to amplify, one was affected (genotype 11.3) and the other was unaffected (genotype 2.3). For the embryo with conflicting results, the healthy genotype 1.2 was clearly demonstrated in four individual blastomeres. Two other blastomeres of this embryo showed ADO: one blastomere showed ADO of allele 1, the other blastomere showed ADO of allele 2. The seventh blastomere failed to amplify.

The efficiency of the first PGD cycle is outlined in Table II. During this PGD cycle no contamination was observed. In total 38 blastomeres clearly containing a nucleus were analysed, 37 of which resulted in amplification, equal to an amplification rate of 97.4%. Twenty-five of these 37 blastomeres were heterozygous and ADO was observed in five of them, giving an ADO rate of 20%. This figure corresponds well to the results obtained previously with research embryos (23.8%).

In a second ICSI cycle, 10 mature oocytes were retrieved, eight of which were fertilized, resulting in five embryos for biopsy. Besides three affected embryos (all three with genotype 11.3, one of which is represented in Figure 2, lane 6), two unaffected embryos (both with genotype 2.3, one is represented in Figure 2, lane 9) were available for transfer. These were two 8-cell embryos at the time of embryo biopsy, one of which

## Table II. Efficiency of the two preimplantation genetic diagnosis (PGD) cycles, including the diagnosis on day 3 (PGD) and check-up of the remaining embryos on day 4

<table>
<thead>
<tr>
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<th>Amplification (%)</th>
<th>Contamination (%)</th>
<th>Allele drop-out (%)</th>
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<tbody>
<tr>
<td><strong>First PGD cycle</strong></td>
<td></td>
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<tr>
<td>PGD (day 3) (5 embryos)</td>
<td>100 (11/11)</td>
<td>0.0 (0/15)</td>
<td>14.3 (1/7)</td>
</tr>
<tr>
<td>Check-up (day 4) (9 embryos)</td>
<td>96.3 (26/27)</td>
<td>0.0 (0/19)</td>
<td>22.2 (4/18)</td>
</tr>
<tr>
<td>Sum</td>
<td>97.4 (37/38)</td>
<td>0.0 (0/34)</td>
<td>20.0 (5/25)</td>
</tr>
<tr>
<td><strong>Second PGD cycle</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGD (day 3) (5 embryos)</td>
<td>100 (8/8)</td>
<td>0.0 (0/8)</td>
<td>0.0 (0/8)</td>
</tr>
<tr>
<td>Check-up (day 4) (3 embryos)</td>
<td>100 (16/16)</td>
<td>20.0 (1/7)</td>
<td>0.0 (0/16)</td>
</tr>
<tr>
<td>Sum</td>
<td>100 (24/24)</td>
<td>6.7 (1/15)</td>
<td>0.0 (0/24)</td>
</tr>
<tr>
<td>Total</td>
<td>98.4 (61/62)</td>
<td>2.0 (1/49)</td>
<td>10.2 (5/49)</td>
</tr>
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</table>

Amplification efficiency and contamination rate are expressed per number of clearly nucleated blastomeres or blank controls analysed. The allele drop-out rate is expressed per number of heterozygous blastomeres. In the first PGD cycle (day 3), four out of eight embryos corresponding to four blastomeres were homozygous. During check-up, confirmation was obtained for three of them (corresponding to eight blastomeres). In the second PGD cycle all five embryos were heterozygous.

During the previous IVF cycle, 4/4 embryos were shown to be affected. In the first PGD cycle, 7/10 embryos displayed the affected genotype. In the second PGD cycle a similar proportion of affected embryos was observed: 3/5 embryos were affected. The overall proportion of affected embryos (14/19, i.e. 73.7%) was considered to be rather high. For this reason, single spermatozoa from the husband were evaluated for their genotype. The sperm parameters were assessed for the sperm sample and were normal: 106×10^6 spermatozoa per ml, 52% progressive motility and 22% normal morphology according to strict Kruger criteria. A similar profile was obtained on the occasion of the two PGD–ICSI cycles. Eighty-five individual spermatozoa were collected and subjected to PCR analysis. Amplification was obtained in 75 spermatozoa (i.e. 88.2%). Only 60 spermatozoa gave clear-cut results, i.e. one single peak of either the duplicated or the healthy allele of the father-to-be. Forty (66.7%) were found to have the duplicated allele 1, whereas only 20 displayed the healthy allele 2 (33.3%). This distribution is significantly different from the theoretical 50% segregation expected for an autosomal dominant trait (χ^2-test, P = 0.0099) and might explain the higher proportion of affected embryos found during both the IVF and ICSI cycles.

### Discussion

To our knowledge, this is the first report of a PCR-based PGD procedure for CMT1A. Molecular diagnosis of the CMT1A
duplicated in blood samples using PCR has been described previously (Blair et al., 1995). Prenatal diagnosis for this disease — based on restriction fragment length polymorphism (RFLP) analysis, short tandem repeat (STR) analysis and multicolour FISH — has also previously been described (Lebo et al., 1993; Navon et al., 1995). However, to avoid abortion of possibly affected pregnancies, diagnosis can be offered at the embryonic stage before implantation. Embryo biopsy of one or two blastomeres from each embryo obtained via IVF with ICSI allows this early form of prenatal diagnosis or PGD.

The assay is based on a polymorphic (CA)n marker localized within the duplication responsible for the disease and for which the couple is informative. Fluorescent PCR and fragment analysis on an automated sequencer was used. This approach results in a high sensitivity at the single-cell level and a high accuracy of sizing the PCR products (Findlay et al., 1996). The latter advantage is of special interest here, because the difference between the amplified fragments of the three alleles is only 4 bp. Analysis on ethidium bromide-stained polyacrylamide gels did not allow a clear separation (data not shown).

The efficiency and accuracy of the assay has been assessed with both EBV-transformed lymphoblasts and blastomeres from research embryos and has been shown to be acceptable for clinical application. We have no explanation for the rather high ADO rate (20 to 25%). High ADO rates observed in the first clinical cycle were similar to the rates observed in the preliminary experiments. In contrast to the first cycle, no ADO was observed in the second cycle (0/8 during the PGD and 0/16 during the check-up the next day), despite the fact that all assay conditions were similar. An alkaline lysis method was used, which is known to result in higher amplification efficiencies than other cell lysis methods (Cui et al., 1989). Efficient separation of the native double-stranded DNA was also ascertained by using 96°C in the 5 min denaturation step (Ray and Handyside, 1996). Due to its high sensitivity, fluorescent PCR allows recognition of some preferential amplifications that are misclassified as ADO in conventional PCR. Indeed, for myotonic dystrophy it has recently been described that when using fluorescent PCR the ADO rate can be significantly lowered as compared to conventional PCR (K.Sermon et al., unpublished observations). Despite the sensitivity of our assay, a considerable ADO rate (10.2%) persists. However, the present PCR approach is designed in such a way that ADO does not lead to transfer of an affected embryo, because the presence of the healthy allele of the father is evaluated for PGD. Only one healthy embryo has been lost for transfer so far due to drop-out of the healthy allele of the father, which could have been avoided only if two nucleated blastomeres had been available for diagnosis.

In total, the genotypes of 19 embryos were evaluated. For two of these, the genotypes were only found at a later stage in pregnancy by means of prenatal diagnosis. The twin pregnancy was terminated because both fetuses were affected. During the same IVF cycle, two embryos were cryopreserved and later donated for research. Both showed the affected genotype. During two ICSI-PGD cycles, 15 embryos were evaluated and only five of them had inherited the healthy allele of the father. During the first cycle, none of the three healthy embryos were available for transfer for the following reasons: one embryo showed unclear results in the two blastomeres, one embryo was lost due to ADO in the single blastomere available for diagnosis and for another embryo no nucleated blastomere was available for diagnosis, resulting in a failure of amplification. Only in the second cycle two healthy embryos were replaced, resulting in a singleton pregnancy. DNA analysis of chorionic villus cells obtained at 10 weeks of gestation indicated the correct genotype as diagnosed during PGD. The pregnancy is ongoing and at the time of proofing is in its 39th week of gestation.

Taken together, the two PGD cycles resulted in only two embryos available for transfer. Successful diagnosis resulting in embryo transfer seems to depend on the availability of two clearly nucleated intact blastomeres at the time of embryo biopsy. When non-conflicting results can be obtained in the two cells, a correct diagnosis is obtained and ADO can be
circumvented. The first PGD cycle did not result in embryo transfer because these criteria were not fulfilled for the few (3/10) healthy embryos present. The second PGD cycle confirms our statement: the healthy genotype was diagnosed in two embryos (two nucleated intact blastomeres were evaluated in each embryo) and resulted in a successful embryo transfer.

The aberrant ratio of affected embryos versus unaffected ones (14/19 embryos were affected, i.e. 73.7%, which is significantly different from the expected 50% segregation in an autosomal dominant trait, $\chi^2$-test, $P = 0.039$) was surprising and therefore investigated further. Single spermatooza of the affected male were analysed for their genotype. A similar distribution, with more spermatooza carrying the affected allele than spermatooza carrying the healthy allele, was observed. Two-thirds of all the analysable sperm cells carried the affected allele. This ratio correlates exactly with 10/15 ICSI embryos being affected and therefore explains the higher proportion of affected embryos. The proposed genetic mechanism causing the CMT1A duplication is unequal non-sister chromatid exchange at meiosis (unequal crossing-over) (Palau et al., 1993). The majority of CMT1A duplications in sporadic patients are of paternal origin, only about 1:8 of the de-novo CMT1A duplications has a maternal origin (Palau et al., 1993; Blair et al., 1996). This observation led to the speculation that male-specific factors may be operating during spermatogenesis that either help in forming the duplication and/or stabilize the duplicated chromosome (Palau et al., 1993). Reiter et al. (1996) have defined a small recombination hot spot of 1.7 kb within the two proximal and distal CMT1A-REP regions. Sequence analysis revealed a mariner transposon-like element (MITE) near the hot spot which might mediate strand exchange events via cleavage by a transposase. The authors propose that the presence of a testis-specific transposase cutting its target site in or around MITE may account for the observation that most of the de-novo CMT1A duplications originate during male meiosis. The patient described here represents a de-novo case of paternal origin. The exact mechanism resulting in the allele duplication during the gametogenesis of the patients’ father remains elusive. The presently observed increased proportion of patients’ spermatooza carrying the CMT1A duplication could suggest that a similar mechanism is operating during his spermatogenesis.

The fluorescent PCR-based test described in this paper provides an efficient and accurate assay for the detection of the CMT1A duplication at the single cell level. On the basis of the presence of the healthy allele of the affected parent-to-be, healthy embryos can be selected for transfer. Results can be obtained within 6 h after biopsy (2.5 h PCR and 3 h ALF gel electrophoresis including interpretation of the results). For these reasons, the assay is suitable for PGD. Informative couples at risk for CMT1A can benefit from the assay because the health status of the embryos is known before implantation, which avoids possible termination of an affected pregnancy. For couples not informative for AFM191, four other CA markers are currently available to detect CMT1A. For each marker (including AFM191), six to nine different alleles can be expected in the overall population. Heterozygosity for these markers varies from 47 to 74%. Informativity for one of these markers — which needs to be evaluated in each individual couple — will allow PGD using one of these alternative markers.

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