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Preimplantation genetic diagnosis for Charcot–Marie–Tooth disease type 1A

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Charcot–Marie–Tooth (CMT) disease is the 'common' name for a range of hereditary peripheral neuropathies. CMT1 is the most common form and is transmitted in an autosomal dominant manner. CMT1A maps to chromosome 17p11.2 and is caused, in the majority of cases, by a 1.5 Mb DNA duplication, that includes the peripheral myelin protein 22 (PMP) gene. This paper reports on preimplantation genetic diagnosis (PGD) for CMT1A in five couples. The CMT1A duplication was detected by fluorescent PCR analysis using polymorphic (CA)n markers localized within the duplication. Single-cell PCR on blastomeres allowed genetic analysis of embryos obtained after ICSI. Only healthy unaffected embryos were transferred to the uterus. PCR experiments with single EBV-transformed lymphoblasts or with research blastomeres allowed the evaluation of amplification efficiencies, as well as contamination and allele drop-out (ADO) rates for each PCR protocol. Three simplex PCR protocols (using one primer pair) and two duplex PCR protocols (using two primer pairs) were developed for CMT1A. Additionally, a protocol using all three primer pairs in triplex was also established. Thirteen clinical ICSI–PGD cycles were performed for five couples (12 simplex PCR cycles and one duplex PCR cycle), resulting in seven embryo transfers. Three singleton pregnancies ensued in two couples and three healthy babies were delivered. This report describes different fluorescent PCR-based tests which allow efficient and accurate single-cell level detection of the CMT1A duplication. On the basis of the presence of the healthy allele of the affected parent-to-be (and/or absence of the affected one), healthy embryos can be selected for transfer. The assays are suitable for PGD for other couples who present with the same CMT1A duplication [depending on their informativity for the (CA)n markers available] as described here.

Key words: Charcot–Marie–Tooth disease/preimplantation genetic diagnosis/single-cell PCR

Introduction

Charcot–Marie–Tooth (CMT) disease, a heterogeneous group of inherited peripheral neuropathies, is one of the most common degenerative neurological disorders with a prevalence rate of 1 in 2500 (Skre *et al*., 1974; Patel and Lupski, 1994). The most common form of CMT, CMT1, is characterized by diffusely low nerve conduction velocities and segmental demyelination on peripheral nerve biopsy (Dyck *et al*., 1993). CMT1 is caused by mutations in one of several genes expressed in Schwann cells, the myelin producing cells of the peripheral nervous system. The majority of patients with CMT1, designated CMT1A, have a 1.5 Mb duplication in the p11-p12 region of chromosome 17, encompassing the entire peripheral myelin protein 22 (PMP22) gene (Hoogendijk *et al*., 1991; Lupski *et al*., 1991). This duplication event accounts for 70–85% of CMT1 cases. A gene dosage effect has been suggested as the mechanism underlying the demyelination neuropathy (Warner *et al*., 1996). The disorder is transmitted in an autosomal dominant manner.

CMT1A is characterized clinically by distal muscle atrophy and weakness, and by a variable degree of motor and sensory impairment, which is usually progressive. Some patients are almost asymptomatic, while others 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).

Molecular diagnosis of CMT1A requires detection of the duplication using DNA probes located in the duplicated region (Latour et al., 2001; Badano *et al*., 2001). Prenatal diagnosis for CMT1A is feasible using this molecular genetic method (Navon *et al*., 1995; Bernard *et al*., 2002) or using multicolour fluorescence *in situ* hybridization (FISH) (Lebo *et al*., 1993; Kashork *et al*., 1999).

The detection of an affected fetus with the option of terminating the pregnancy can constitute a difficult dilemma for the parents-to-be. A possible alternative in these cases is preimplantation genetic diagnosis (PGD) (Handyside *et al*., 1998; Harper and Wells, 1999; Sermon, 2002). This very early form of prenatal diagnosis performed on embryos obtained through IVF–ICSI offers couples at risk the possibility of selecting unaffected embryos for transfer and thus of avoiding termination of pregnancy. Embryos are obtained by IVF with ICSI and their disease status is determined by DNA analysis of one or two blastomeres biopsied from each embryo. PGD for CMT1A was first reported by us in 1998 (De Vos *et al*., 1998). Detection of the CMT1A duplication in single blastomeres was done through fluorescent PCR with a polymorphic (CA)n marker localized within the duplication. Genomic DNA of the patient and his parents was analysed to allow identification of the affected and unaffected alleles. PCR

Table I. Primer sequences of the three (CA)n markers

Locus	Marker	Sequence	Fragment length (bp)
D17S921	AFM191	xh12a 5′-*CTTGGACTCCTACAAATCCTGGCA-3′ xh12m 5′-GGCCACCATAATCATGTCAGACAAT-3′	174–182
D17S261	Mfd41	5′-CAGGTTCTGTCATAGGACTA-3′ (CA strand) 5′-*TTCTGGAAACCTACTCCTGA-3′ (GT strand)	158–162
D17S122	RM11	5′-GGCCAGACAGACCAGGCTCTGC-3′ (CA strand) 5′-*CAGAACCACAAAATGTCTTGCATTC-3′ (GT strand)	158–164

The asterisks indicate the Cy5 (indocyocarbenine) label on one of the two primers.

amplification of genomic DNA of the parents-to-be, followed by automated laser fluorescence (ALF) electrophoresis of the amplified fragments showed that the unaffected allele of the patient was distinct from the alleles of his partner. The presence of this unaffected allele of the father-to-be in embryos indicated healthy embryos (De Vos *et al*., 1998).

The present report represents an extension of our previous work (De Vos *et al*., 1998) and describes novel PCR-based assays developed for the detection of the CMT1A duplication using two additional polymorphic (CA)n markers. The efficiency and accuracy of these tests was evaluated preclinically. Prior to clinical application the segregation phase of the marker(s) with the duplication was determined either on spermatozoa (for one couple) or on oocytes and polar bodies (PBs) (for two couples). In a further two couples, this information was derived from genomic DNA analysis of the patient and his/her parents. Clinical application involved 13 cycles for five couples where one partner carried the mutation. Using these assays, unaffected embryos were selected for transfer and three pregnancies resulted.

Materials and methods

Collection of single cells

Preliminary PCR experiments were performed both on Epstein–Barr-virus transformed lymphoblasts (representing a continuous cell source) and on 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. The collection of these cells has been described elsewhere (Sermon *et al*., 1998).

Single sperm cells were collected from one male CMT1A patient in order to determine the segregation phase of the AFM191 marker with the duplication. The sperm sample was prepared using a two layer Pure Sperm 90–45% density gradient (Nidacon International AB, Göteborg, Sweden). After centrifugation (20 min at 300 *g*), 200 µl of the pellet was carefully aspirated. The pellet was washed twice with HEPES-buffered Earle's medium (5 min at 750 g) and the final pellet was resuspended in ~100 µl of medium. A small fraction of the sperm sample (1–2 µl) was added to a 5 µl droplet of 10% (w/v) polyvinylpyrrolidone (PVP; Sigma Aldrich) in HEPES-buffered Earle's medium under oil. Sampling of single sperm cells was performed on an inverted microscope (Diaphot, Nikon Corporation, Tokyo, Japan) at 400× magnification using Hoffman Modulation Contrast (Modulation Optics, Inc., Greenvale, New York, NY, USA) and a microinjection pipette. Single motile sperm cells were immobilized one by one and washed twice in two separate washing droplets of Earle's medium with PVP. They were then transferred to single 0.2 ml PCR tubes containing 2.5 µl of alkaline lysis buffer (ALB) (De Vos *et al*., 1998). After each sperm cell, a blank control was taken by expelling some medium from the injection pipette into an ALB-containing PCR tube.

ICSI procedure

Ovarian stimulation was carried out by pituitary desensitization with gonadotrophin-releasing-hormone analogues (buserelin; Suprefact®; Hoechst, Brussels, Belgium) combined with human menopausal gonadotrophins (Humegon®; Organon, Oss, The Netherlands) or recombinant FSH (Gonal-F®; Serono, Brussels, Belgium, or Puregon®; Organon). Human chorionic gonadotrophin (HCG) (10 000 IU; Pregnyl®; Organon, or Profasi®; Serono) was administered when at least three follicles of 17 mm diameter were seen on a vaginal ultrasound scan. Transvaginal ultrasound-guided oocyte retrieval was scheduled 36 h after HCG administration. The luteal phase was supplemented daily by 600 mg of micronized progesterone, administered intravaginally (Utrogestan; Piette, Brussels, Belgium). ICSI rather than IVF was used to fertilize the oocytes to prevent residual contamination with sperm (Liebaers *et al*., 1998). The ICSI procedure on mature metaphase-II oocytes was performed as described by Joris *et al*. (1998). Fertilization was examined ~16–18 h after ICSI. Embryo culture was performed in sequential media, either G1.1/G1.2 or G2.1/G2.2 (Vitrolife, Göteborg, Sweden), CCM/CBM (Cook, Brisbane, Australia) or Medicult medium (Copenhagen, Denmark).

Polar body biopsy and cleavage-stage embryo biopsy

PB biopsy involved the removal of the first PB on the day of ovum pick-up by opening of the zona pellucida by laser drilling (Fertilase; MTM Medical Technologies Montreux, Switzerland; Sermon *et al*., 1998) and aspiration of the PB. PB biopsy was only used to determine the segregation phase of the AFM191 marker with the CMT1A duplication in the oocytes of one female patient.

Embryo biopsy was carried out in the morning of day 3 after oocyte microinjection. Calcium–magnesium-free medium EB10 (Scandinavian IVF Science, Göteborg, Sweden) was used for decompaction of the embryos prior to biopsy (5–10 min incubation). Laser technology (Fertilase) was used to drill a hole in the zona pellucida (on average two to three pulses of 7 ms were applied). Two blastomeres per embryo (⩾6 cell stage) containing a clear nucleus were gently aspirated through the hole. After biopsy, the blastomeres were washed three times in calcium–magnesium-free medium (containing 0.4% bovine serum albumin) and transferred to a 200 µl PCR tube containing 2.5 µl of ALB (De Vos *et al*., 1998). KOH-containing ALB (200 mmol/l KOH, 50 mmol/l DTT) was used for PCR with AmpliTaq polymerase (Perkin Elmer) and NaOH-containing ALB (200 mmol/l NaOH, 50 mmol/l DTT) was used for PCR with an Expand High Fidelity (EHF) kit (Roche, Brussels, Belgium), because potassium may have an inhibitory effect on EHF polymerase. The tubes were kept at –80°C for at least 30 min. Cell lysis and neutralization with neutralization buffer has been described elsewhere (De Vos *et al*., 1998). In cases where the EHF kit was used, neutralization was performed by adding 2.5 µl of 200 mmol/l Tricine pH 4.9.

PCR procedures for CMT1A detection

For CMT1A detection, three different (CA)n markers located within the duplication were used: AFM191×h12a*/m located at locus D17S921 (Gyapay *et al*., 1994; De Vos *et al*., 1998), Mfd41GT*/CA located at locus D17S261 (Weber et al., 1990; Navon *et al*., 1995) and RM11GT*/CA located at locus D17S122 (Lupski *et al*., 1991; Navon *et al*., 1995). The asterisks indicate the presence of a Cy5 (indocyocarbenine) label on one of the two primers (Eurogentec, Seraing, Belgium or Amersham Pharmacia Biotech, Roosendaal, The Netherlands). For primer sequences, see Table I.

Three simplex PCR protocols were developed, i.e. one for each of the markers. The protocol for the AFM191 marker has been described before (De Vos *et al*., 1998). Two small modifications were adopted: the number of PCR cycles was modified (47 instead of 45 cycles) due to the use of a Cy5 labelled forward primer (instead of a fluorescein labelled one, which was used on the older version of the ALFExpress, the ALF) and decontamination of the reaction

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Table II. Clinical characteristics of the five couples prior to their PGD cycles for CMT1A and post-PGD results

Couple	Patient	Female age	Mutation origin	Reproductive history Pre-PGD		Post-PGD
1	Male	31	Novel	G2P0A2	Idiopathic infertility → IVF A1, spontaneous A2, TOP post-CVS (CMT1A) (affected twin pregnancy)	PGD 2: pregnancy, PND, healthy girl now 4 years PGD 3: pregnancy, PND, healthy boy now 2 years
2	Female	30	Paternal	G0P0A0	No fertility problem PGD because objection to abortion	PGD 3: pregnancy, no PND, healthy boy now 7 months
3	Male	31	Novel	G2P0A2	No fertility problem 2× TOP post-CVS (CMT1A)	PGD 1–2: no pregnancy Future: undecided
4	Female	33	Novel	G0P0A0	No fertility problem PGD because objection to abortion	PGD 1–4: no pregnancy Future: PGD 5
5	Female	28	Novel	G0P0A0	No fertility problem PGD because objection to abortion	PGD 1: no pregnancy

G = gestation; P = partus (i.e. delivery); A = abortion; TOP = termination of pregnancy; CVS = chorion villus sampling; PND = prenatal diagnosis.

mix involved ScrfI (instead of NlaIII). The protocol for the Mfd41 marker was similar to the AFM191 protocol, except for HinfI, which was used for decontamination of the reaction mix, and for the annealing temperature during PCR (10 + 35 cycles) which was 52°C. In the first 10 cycles the denaturation temperature was 96°C, whereas in the following 35 cycles it was 94°C. For the RM11 marker, the concentration of both primers was 0.4 µmol/l and 1.4 IU EHF DNA polymerase (Roche) was used in combination with reaction buffer 2 supplied within the EHF kit. Decontamination of the reaction mix was carried out with ScrfI. The PCR programme was as follows: 5 min denaturation at 95°C, followed by 44 cycles of 30 s at 95°C, 30 s at 62°C, 45 s at 72°C, followed by 7 min at 72°C.

The AFM191–Mfd41 duplex involved amplification with EHF DNA polymerase as described above. The concentration of the AFM191 primers and the Mfd41 primers was 0.1 and 0.4 µmol/l respectively. The reaction mix was not decontaminated. The PCR programme involved 47 cycles using an annealing temperature of 55°C.

For the AFM191–RM11 duplex, amplification was performed with AmpliTaq polymerase (Perkin Elmer). The concentration of the primers was 0.1 and 0.4 µmol/l for AFM191 and RM11 respectively. The reaction mix was decontaminated with NlaIII and ScrfI. The two primer sets were used simultaneously in one PCR without separating. The PCR programme involved 10 + 40 cycles (denaturation temperature respectively 96 and 94°C) and the annealing temperature was 62°C.

For the triplex approach, using all three primer sets together, EHF DNA polymerase was used for amplification (0.875 IU in the first round and 1.4 IU in the second rounds). The concentration of the primers were 0.2, 0.3 and 0.2 µmol/l for AFM191, Mfd41 and RM11 respectively in the two rounds. The reaction mix was not decontaminated. The PCR program of the first round involved 5 min denaturation at 95°C, followed by 10 cycles of 30 s at 95°C, 30 s at 55°C, 45 s at 72°C, followed by 7 min at 72°C. Then, the reaction was split into two separate PCRs, using 3 µl of the first PCR product in the second rounds. In one second round the AFM191 and Mfd41 primer sets were used together, whereas in the other second round the RM11 primer set was used separately. Annealing temperatures were 55 and 60°C respectively. The number of cycles was 35 and 32 respectively. PCRs were carried out on a Perkin Elmer Cetus GeneAmp PCR System 2400 or on an Eppendorf Mastercycler Gradient (VWR International, Leuven, Belgium). PCR products (3 µl) were mixed with 3 µl of loading buffer (0.0125 g bromophenol blue, 2 ml glycerol, 3 ml H_2O) and loaded onto an ALFExpress Automated DNA Sequencer from Amersham Pharmacia Biotech (Roosendaal, The Netherlands). Results were processed using Allelelinks software provided by the manufacturer (De Vos *et al*., 1998).

Patient description

Five couples requested PGD for CMT1A (Table II). Couple 1, where the male partner carried the CMT1A duplication, has been described before (De Vos *et al*., 1998). The mutation in the patient who presented with mild symptoms, represented a novel mutation. In couple 2, the CMT1A duplication was detected in the female partner using PCR analysis with (CA)n polymorphic markers (Navon *et al*., 1995) located within the duplication. The patient, who presented with minimal symptoms, had inherited the mutation from her affected father. This couple opted for PGD because of objections to abortion in case an affected pregnancy is detected at prenatal diagnosis. In couple 3 the CMT1A duplication in the male partner, who presented with minimal symptoms, was a novel mutation. Therefore, the segregation phase of the duplication with the polymorphic (CA)n marker had to be determined on single sperm cells of the patient prior to clinical PGD. The couple had had two previous abortions of affected pregnancies detected at prenatal diagnosis and therefore now opted for PGD. Couple 4 also involved a novel mutation, but in the female partner. Objection to abortion of a possibly affected pregnancy was their reason for opting for PGD. Analysis of the segregation phase of the mutation with the polymorphic (CA)n marker was on first PBs and oocytes of the patient prior to clinical PGD. In couple 5 the female partner carried a *de novo* CMT1A duplication. A similar segregation analysis as for couple 4 was carried out on the patient's first PBs and oocytes. The couple opted for PGD in order to avoid abortion in case of an affected pregnancy. Initially showing interest in CMT1A-PGD, a sixth couple decided not to go through with PGD (therefore not included in Table II).

Results

CMT1A detection using polymorphic (CA)n markers

Five couples requested PGD for the CMT1A duplication. Two couples (couples 3 and 4) were found to be only informative for one of the three markers. The genotypes of the parents-to-be were determined through electrophoresis of the amplified fragments. The identification of the affected and unaffected alleles allowed diagnosis (Table III). For PGD, the presence of the healthy allele of the patient (in the absence of the duplicated one) was evaluated.

Couple 1

DNA analysis for couple 1 was carried out using Southern blot analysis of markers recognizing restriction fragment length poly-

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Table III. Genotypes of the five couples requesting PGD for CMT1A (respectively, two male carriers and three female carriers) and the corresponding ALF patterns

Couple	Marker	Couple's genotypes (female and male respectively)		ALF pattern (bp)	Unaffected patterns (bp)	Affected patterns (bp)
1	AFM191	F	*1.3*	183–175	183–179	183–183
		M	*11.2*	183–183–179	179–175	183–175
2	Mfd41	F	*13.3*	162–158–158	160–158	162–158–160
		M	*2.3*	160–158	158–158	162–158–158
3	AFM191	F	*2.3*	180–176	180–174	182–174–180
		M	*14.4*	182–174–174	176–174	182–174–176
4	RM11	F	*1.34*	164–160–158	164–162	162–160–158
		M	*2.2*	162–162	164–162	
5	AFM191	F	*14.4*	182–174–174	174–176	182–174–176
		M	*3.3*	176–176		
	Mfd41	F	*1.33*	162–158–158	162–158	158–158–158
		M	*3.3*	158–158		

The healthy allele of each patient is underlined. The segregation phase of the second male carrier (couple 2) was determined on single spermatozoa of the patient. The segregation phase of the second female carrier (couple 4) was determined on her oocytes using first PB biopsy. The ALF patterns of future unaffected and affected embryos were deduced. For PGD, the presence of the healthy allele of the patient is evaluated (couple 1 and couple 4). For couples 2 and 3, the absence of allele 1 indicates a healthy genotype. For couple 5, the presence of allele 1 for the Mfd41 marker (162 bp) and the absence of allele 1 for the AFM191 marker (182 bp) indicates a healthy genotype.

morphisms and CA repeat polymorphisms located in the CMT1A duplication (De Vos *et al*., 1998). Analysis included genomic DNA of the couple and of the patient's parents.

Couple 2

Genomic DNA of the female patient, her parents and her partner was analysed for the presence of the CMT1A duplication, using PCR analysis with (CA)n markers located within the duplication (Mfd41, RM11 and AFM191). The patient was homozygous for the duplication haplotype for the markers RM11 and AFM191. The Mfd41 marker was found to be suitable for PGD (Table III): if allele 1 is present in the embryo it carries the duplication.

Couple 3

For the male patient of couple 3 (Table III), the segregation phase of the AFM191 marker with the duplication was unknown and therefore determined on single sperm cells. Twenty single spermatozoa were collected and analysed and 17 out of 20 amplified. Two of the 20 blank controls amplified, which was probably due to erroneous spermatozoon transfer to the blank control tube, since the corresponding sample tube signalled no amplification signal in either case. A single peak at 174 bp (i.e. allele 4) was found amplified from eight spermatozoa, whereas eight other spermatozoa resulted in two products, a 174 bp product (allele 4) and a 182 bp product (allele 1). From these results, the genotype *14.4* (instead of *1.44*) could be deduced. One spermatozoon yielded a single 182 bp peak, suggesting that the 174 bp fragment did not amplify.

Couple 4

Because the female patient of couple 4 (Table III) was an isolated case within her family, the segregation phase of the RM11 marker with the duplication had to be determined on her oocytes and PBs. In a first stimulation cycle, five oocytes were available. It was agreed with the patient that if fewer than six oocytes were available, they would all be used for genetic analysis. The five first PBs were successfully biopsied and thus separated from their corresponding oocytes. One PB and corresponding oocyte resulted in no amplification. In another PB and its corresponding oocyte all three alleles were present. From the remaining three PBs the genotype *1.34* (instead of *13.4* or *14.3*) was deduced. However, two oocytes showed contamination with the alleles residing in the corresponding PB. In the couple's first PGD cycle, eight first PBs were removed from the eight metaphase-II oocytes available, just before microinjection of the oocytes. Confirmation of the previously deduced genotype was obtained. Four PBs showed absence of the duplication, which therefore resided in the corresponding oocytes, and three PBs showed the duplication, which means that the corresponding oocytes were devoid of the mutation. One PB showed the presence of all three peaks (indicating, in this particular case, that crossover had occurred and the genotype of the oocyte could not be deduced). Six out of eight oocytes were fertilized, although only three embryos were available for biopsy. Analysis of the blastomeres confirmed the PB results for two embryos (one affected and one unaffected, which was transferred to the patient). The third embryo was revealed to be healthy but did not divide further and was therefore not suitable for embryo transfer.

Couple 5

For couple 5 (Table III), who wanted PGD for CMT1A, a duplex PCR approach (AFM191–Mfd41) was established. Genotyping of the female carrier showed alleles 1, 4 and 4 for the AFM191 marker and alleles 1, 3 and 3 for the Mfd41 marker. The segregation phase of the duplication with the markers also had to be determined by means of PB biopsy, because of the *de novo* character of the mutation. The male partner was homozygous for both markers (*3.3* for AFM191 and *3.3* for Mfd41). In the patient's stimulation cycle, 34 metaphase-II oocytes were available. Twelve first PBs were successfully biopsied and thus separated from their corresponding oocytes. PCR analysis of these PBs (the corresponding oocytes were used in the patient's ICSI–PGD cycle) gave the following results: five out of 12 showed affected PBs (alleles 1 and 4 for the AFM191 marker and a duplicated allele 3 for the Mfd41 marker) and two out of five showed unaffected PBs (allele 4 for the AFM191 and allele 1 for the Mfd41 marker). The remaining five PBs were heterozygous due to crossover. For three homozygous PBs, confirmation of the diagnosis was obtained in the embryos

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Table IV. Amplification, allele drop-out (ADO) and contamination rates for the different PCR protocols for CMT1A (three simplex, two duplex and one triplex protocol) as evaluated in pre-clinical experiments on single EBV-transformed lymphoblasts

PCR protocol	Cell type	Amplification % (numbers)	ADO % (numbers)	Contamination % (numbers)
AFM191	L	91.4 (32/35)	25.0 (8/32)	0.0 (0/35)
	B	97.7 (42/43)	11.8 (4/34)	3.8 (1/26)
Mfd41	L	94.3 (33/35)	3.0 (1/33)	0.0 (0/35)
RM11	L	93.8 (45/48)	2.2 (1/45)	4.1 (1/24)
AFM191 + Mfd41	L	92.0 (46/50)	0.0 (0/46)	2.0 (1/50)
AFM191 + RM11	L	93.8 (15/16)	0.0 (0/15)	0.0 (0/8)
Triplex – AFM191	L	92.3 (48/52)	0.0 (0/48)	0.0 (0/52)
Triplex – Mfd41	L	86.5 (45/52)	0.0 (0/45)	0.0 (0/52)
Triplex – RM11	L	94.2 (49/52)	0.0 (0/49)	0.0 (0/52)

The pre-clinical results for the AFM191 protocol have been published elsewhere (De Vos *et al*., 1998), post-PGD results on blastomeres from two cycles are mentioned as well in order to show improvement in the ADO result (ADO can only be assessed on the heterozygous cells, 34 out of 42 amplified). For each of the two duplex PCR protocols, identical amplification and ADO results were obtained for the two markers. L = lymphoblast; B = blastomere.

resulting from the corresponding oocyte, i.e. the complement genotype of the PB was found. From the corresponding oocytes of the other four homozygous PBs no embryos suitable for biopsy were obtained and thus no analysis was done.

A sixth couple for CMT1A-PGD decided not to go through with PGD at the time the duplex PCR protocol (AFM191–RM11) was established. Therefore, no clinical application of this test has yet been performed.

Efficiency and accuracy of the different tests

The efficiency and accuracy of the different PCR protocols was evaluated in preliminary experiments on single EBV-transformed lymphoblasts with the known genotype of the parents-to-be or on research embryo blastomeres (Table IV). PCR on single lymphoblasts resulted in amplification rates ranging from 86.5 to 94.3%, whereas only three of the blank controls (204 in total) amplified (i.e. 1.5%). The lowest amplification rate was obtained for the Mfd41 marker in the triplex approach. Allele drop-out (ADO) rates ranged from 0 to 25%. For the AFM191 protocol, results on post-PGD blastomeres from two cycles are also documented, showing a considerable improvement in ADO rate (11.8%) compared with the results obtained with lymphoblasts. Using the AFM191 marker in a duplex PCR, AFM191–RM11 or AFM191–Mfd41, under slightly different conditions (more cycles and different annealing temperature), resulted in no ADO. Similarly, no ADO was observed for any of the three markers in the triplex PCR.

Interestingly, when analysing the cells from patient 4 with three different alleles for RM11, no more ADO than in cells with only two different alleles was observed (1/45, i.e. 2.2%).

Clinical application

Thirteen ICSI–PGD cycles were carried out for five couples (Table V). In total, 138 cumulus–oocyte complexes were retrieved, corresponding to 112 mature metaphase-II oocytes (i.e. a mean of 8.6 per cycle). An overall fertilization rate of 81.9% per injected metaphase-II oocyte was obtained. One cycle remained without fertilization (only one oocyte was available for microinjection). Fifty-one normally fertilized oocytes yielded embryos suitable for biopsy of two blastomeres (i.e. ⩾6 cell stage, ⩽50% anucleate fragmentation) on the morning of day 3 (i.e. 53.7% per 2-PN or normally fertilized oocytes). In seven embryos [4 or 5 cell stage or derived from *in vitro* matured (IVM) germinal vesicle (GV) stage oocytes, four in the first cycle], only one blastomere was removed. This was during the early cycles, when we were still willing to perform a diagnosis on a single blastomere. Subsequently, it was decided to establish a diagnosis on the basis of two intact blastomeres. Removal of an extra cell occurred seven times because a cell had lysed (in three embryos) or a cell had been lost during biopsy (in one embryo) or when being transferred to the PCR tube (three cells from two embryos). Four other blastomeres were not suitable for PCR analysis (one showed no nucleus and three represented a lysed cell, although no extra cell was removed in these cases). The biopsy lysis rate was thus 5.2% (six out of 116 cells removed). PCR analysis was possible on two cells for 47 embryos and on one cell for 11 embryos. Cells for analysis were clearly nucleated. One cycle (with only one fertilized oocyte) remained without biopsy due to poor embryo quality on day 3.

An overall amplification rate of 97.1% (102 out of 105 amplified) was obtained during the PGD. None of the blank controls signalled positive (0/105). The overall ADO rate (on 95 heterozygous blastomeres) was 1.1%.

Eight embryos resulted in no diagnosis (i.e. 13.8%), for several reasons: no amplification in a single blastomere available (two embryos, resulting from IVM-GVs), unclear and conflicting results between two blastomeres (two embryos) or because diagnosis of an unaffected embryo was not established in one blastomere (two embryos). One embryo (PGD cycle of couple 5) showed the presence of the healthy maternal alleles in two blastomeres, but no paternal alleles were present. One embryo (PGD cycle of couple 5) showed the presence of a supernumerary peak for one marker (the other marker was not informative on this matter) in both blastomeres. Post-PGD the same result was obtained, suggesting trisomy of chromosome 17 or triploidy of the embryo. The embryos without diagnosis were analysed again post-PGD: two affected and four unaffected embryos were found, whereas for two embryos again no diagnosis was obtained. Overall, an affected/unaffected embryo ratio of 32/24 was obtained. All 30 affected embryos were available for post-PGD analysis: 29 were confirmed to be affected, whereas only one yielded a doubtful result. Four unaffected embryos were unsuitable for transfer or cryopreservation. They were available for post-PGD analysis and were confirmed to be unaffected.

Eleven cycles with biopsy resulted in seven transfer cycles. In four cycles, no embryo transfer was possible because no unaffected embryos were available. Overall, 11 embryos were transferred (i.e. a mean of 1.5 per patient). In one cycle, three unaffected embryos were cryopreserved and in another cycle two unaffected embryos were

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Table V. Clinical results of 13 PGD cycles (including one cycle for a second child)

Couple	Test	COC	MII	ICSI	2-PN	No. of biopsied embryos	No. of analyzed blastomeres	No. of blastomeres with amplification	No. of blastomeres with ADO[a]	No. of embryos without diagnosis	No. of affected embryos	No. of unaffected embryos	No. of embryos transferred	Outcome
1	AFM191	11	7	11	10	10	13	11	1/7	3	7	0	–	–
1	AFM191	11	10	10	8	5	8	8	0/8	0	3	2	2	Singleton delivery
1	AFM191	11	7	7	7	6	11	11	0/10	1	1	4	1	Singleton delivery
2	Mfd41	8	8	8	6	1	2	2	0/2	0	1	0	–	–
2	Mfd41	10	8	8	6	2	4	4	0/2	0	0	2	1	NP
2	Mfd41	12	12	12	11	7	14	13	0/5	1	3	3	3	Singleton delivery
3	AFM191	3	1	1	0	–	–	–	–	–	–	–	–	–
3	AFM191	5	4	4	3	3	6	6	0/6	0	3	0	–	–
4	RM11	8	8	8	6	3	6	6	0/6	0	1	2	1	NP
4	RM11	3	3	3	1	0	–	–	–	–	–	–	–	–
4	RM11	6	6	6	3	2	4	4	0/4		1	3	1	NP
4	RM11	4	4	4	3	2	4	4	0/4		2	0	–	–
5	AFM191[b] Mfd41[b]	46	34	34	31	17	33	33 33	0/30 0/11	3	8	6	2	NP
Total		138	112	116	95 81.9%	58 61.1%	105	102 97.1%	1/95 1.1%	8 13.8%	30	20	11	

[a]ADO on heterozygous blastomeres only.
[b]Duplex AFM191–Mfd41, with amplification and ADO rates respectively, for the AFM191 and the Mfd41 marker.
COC = cumulus–oocyte complex; MII = metaphase-II oocyte; NP = not pregnant.

available for cryopreservation. Transfers were mainly performed on day 5, one transfer cycle was on day 3 (the earliest) and one was on day 4. Three cycles resulted in a pregnancy for two patients. In two pregnancies (couple 1), PGD was confirmed by chorion villus sampling (CVS). One patient (couple 1) delivered two healthy singleton babies, whereas one patient (couple 2) delivered one healthy singleton. All three babies were born at term after an uncomplicated pregnancy and a normal vaginal delivery. Their birthweight, length and head circumference were in the normal range. The PGD results represent a delivery rate of 42.9% per transfer cycle, 27.3% per cycle with biopsy or 23.1% per started PGD cycle.

Discussion

PGD for CMT1A has been reported before (De Vos et al., 1998). In this study, we extended the number of fluorescent PCR protocols using two additional polymorphic (CA)n markers, RM11 and Mfd41, which allowed the treatment of other couples not informative for the AFM191 marker previously used.

Fluorescent PCR and fragment analysis of amplified fragments on an automated sequencer was used. This approach results in high sensitivity at the single-cell level (Sermon, 2002). It was successfully used for segregation phase analysis on single spermatozoa, oocytes and PBs, and allowed a clear distinction between an affected and healthy genotype in single blastomeres. The efficiency and accuracy of the assays has been assessed with EBV-transformed lymphoblasts (or blastomeres from embryos donated for research) and has been shown to be acceptable for clinical application. Despite the sensitivity of the assays, a considerable ADO rate (11.8%) persisted for the AFM191 simplex protocol. However, the PCR approach for the first couple was designed in such a way that ADO did not lead to transfer of an affected embryo, because the presence of the healthy allele of the affected parent-to-be is evaluated for PGD. Nevertheless, the ADO rate for the AFM191 simplex PCR during clinical application was more favourable (1/37 or 2.7%), indicating that differences according to cell type (lymphoblast or blastomere) do exist. Intriguingly, no ADO was observed when using the AFM191 marker in duplex or triplex PCR, which involved slightly different PCR conditions. These small modifications could be adopted in the simplex protocol if necessary.

For couples 1 and 4, ADO does not lead to the transfer of an affected embryo. However, for couples 2 and 3, ADO of allele 1 may lead to a diagnostic error. Because duplex PCR was not possible for these couples (due to non-informativity for the other two markers), two-cell biopsy was performed in order to detect possible ADO. The result in one blastomere can thus be confirmed by the result of the second blastomere. For couple 5, ADO of allele 1 (AFM191 marker) may also lead to misdiagnosis, however here, this event would be detected by the Mfd41 marker used in duplex.

In total, 58 embryos were analysed in 13 PGD cycles. Embryo biopsy was very efficient with a lysis rate as low as 5.2%. Amplification, contamination and ADO rates were similar to or better than the pre-clinical tests.

A 'no diagnosis' rate of 13.8% was obtained. However, given that two nucleated intact blastomeres were available, diagnosis was obtained in 93.1% of the embryos analysed. Whenever doubt or conflicting results between two blastomeres occurred, we refrained from diagnosis in order to avoid the transfer of a possibly affected embryo. Additionally, diagnosis of an unaffected embryo was never based on one blastomere if the second blastomere showed no amplification. Lysed cells were not used for diagnosis either. Although this resulted in the loss of healthy embryos (albeit only three out of 58), it more importantly avoided the transfer of possibly affected embryos. Post-PGD analysis on 34 embryos did not reveal any misdiagnosis.

Today, the discussion still remains as to whether the removal of two cells from a ⩾7 cell stage embryo reduces its capacity to implant compared with when only one cell is removed (Van de Velde *et al.*, 2000; De Vos and Van Steirteghem, 2001). Only prospective randomized trials will be able to fully address this issue. Of prime concern is that the diagnosis should be safe, i.e. accurate and efficient. Misdiagnosis (five reported so far) is not acceptable. Analysing two blastomeres from the embryo increases the accuracy and reliability of the diagnosis. However, an alternative way to reduce misdiagnosis is

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the development of multiplex PCR protocols using linked informative polymorphic markers. Today, multiplex PCR, with or without detection by fluorescent PCR, is becoming standard, especially for the detection of possible ADO and contamination (Sermon, 2000), thereby avoiding misdiagnosis. Two such duplex PCR protocols for CMT1A were established here and are thus available for clinical application. The development of the triplex protocol has the additional advantage that the test can be used for virtually all couples at risk for CMT1A, provided they are informative for at least two markers (Sermon, 2000). Amplification efficiencies obtained in the triplex PCR are not lower as compared with the duplex or simplex approach (except for the Mfd41 marker). Lower amplification efficiencies in multiplex PCR (due to their complexity) are counter-balanced by the fact that contamination and ADO can be detected. Recently, additional polymorphic markers have been described for CMT1A diagnosis (Latour *et al*., 2001; Badano *et al*., 2001). Heterozygosity for these markers varies from 59 to 93%. Informativity for these markers needs to be evaluated in each individual couple. However, the availability of more polymorphic markers increases the possibility of future duplex and/or multiplex developments, so that more CMT1A couples can be treated with the same protocol. Ideally, one multiplex assay for all CMT1A requests would represent an important time-saving advantage over individual protocols for each individual couple, because establishment of the assay is the most time-consuming part of PGD.

Different fluorescent PCR-based tests for CMT1A PGD are described in this paper. They are all suitable for clinical application and have been applied in 13 treatment cycles for five couples. An acceptable delivery rate of 42.9% per transfer, 27.3% per cycle with biopsy and 23.1% per started cycle is reported. Other couples presenting the same CMT1A mutation can benefit from genetic analysis at the single-cell level before implantation, thereby avoiding termination of an affected pregnancy.

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