Successful PGD for late infantile neuronal ceroid lipofuscinosis achieved by combined chromosome and TPPI gene analysis

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Abstract Late infantile neuronal ceroid lipofuscinosis (NCL-2) is a severe debilitating autosomal recessive disease caused by mutations in TPPI. There are no effective treatments, resulting in early childhood death. A couple with two affected children presented for reproductive genetic counselling and chose to undertake IVF and preimplantation genetic diagnosis (PGD) to avoid the possibility of another affected child. However, DNA testing revealed only one mutation in the proband inherited from mother. Linkage analysis identified five informative linked short tandem repeat markers to aid the genetic diagnosis. Following IVF, five cleavage-stage embryos were biopsied and blastomeres were first subjected to whole-genome amplification, then a series of downstream molecular genetic analyses to diagnose TPPI genotype and finally array comparative genomic hybridization (CGH) to assess the chromosomal ploidy of each embryo. Two unaffected euploid embryos were identified for transfer. One was transferred on day 5 resulting in an ongoing pregnancy. Confirmatory prenatal diagnosis by amniocentesis showed concordance of the embryo and fetal diagnosis. As far as is known, this is the first successful report of PGD for NCL-2 using double-factor PGD with simultaneous single-gene testing and array CGH to identify an unaffected and chromosomally normal embryo for transfer.

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Introduction

Inheritance of serious genetic conditions that have plagued families for many generations can be avoided by the application of IVF and preimplantation genetic diagnosis (PGD) (Simpson, 2010). With advances in knowledge of genetic variation such as short tandem repeats (STR) and single-nucleotide polymorphisms (SNP) across the human genome together with the development of more robust single-cell whole-genome amplification (WGA) techniques (Fiorentino, 2012), it is now possible to diagnose any single-gene disorder in a patient’s embryos where the molecular cause is known. Furthermore, advances in total chromosome analysis by array comparative genomic hybridization (CGH) (Helli et al., 2008) can also provide useful information about the viability status of the unaffected embryos for transfer, increasing the potential success of single-gene PGD cycles. PGD for rare genetic autosomal recessive conditions are commonly requested, but continue to remain a diagnostic challenge for the laboratory in situations where there is lack of genetic information of the family pedigree. For example, even after DNA testing involving exonic gene sequencing, sometimes only one of the two causative mutations are identified and in some cases, neither mutation can be found, presumed to reside in intronic or regulatory sequences. Generally, additional sequencing of intronic and regulatory regions of the gene is not an option due to the paucity of laboratories specializing in the genetics of rare diseases and the high cost to the patient. In these cases, successful PGD is very dependent on the identification of informative polymorphic STR traced through the respective family members or affected children to serve as the primary diagnostic gene markers.

One such rare autosomal recessive disease is late infantile neuronal ceroid lipofuscinosis (LINCL), also known as NCL type 2 (NCL-2). NCL-2 is caused by a genetic deficiency of the lysosomal acid protease tri-peptidyl-peptidase 1 (TPP1) resulting in lysosomal dysfunction (Chang et al., 2012; Sleat et al., 1999). TPP1 is located at 11p15 on chromosome 11 and contains 13 exons (Liu et al., 1998). Through DNA testing of affected families, 89 TPP1 variants involving missense, nonsense, splice site affecting and deletion mutations have now been identified (Ju et al., 2006; Kousi et al., 2012) (http://www.ucl.ac.uk/ncl/mutation). Onset of NCL-2 clinical symptoms typically occurs at 2–4 years of age and is characterized by loss of muscle coordination, seizures, visual impairment and progressive mental deterioration (Ju et al., 2006). Life expectancy is generally between 6 and 15 years of age and there are no effective treatments. To prevent the birth of children with NCL-2, prenatal diagnosis was originally performed by electron microscopy to detect pathogenic lysosomal storage inclusions in chorion villous samples (Goebel, 1994). There has only been one report of prenatal diagnosis for NCL-2 by molecular testing for mutations in TPP1, where four pregnancies from three couples confirmed conception of unaffected fetuses (Zhong et al., 2005). Here is reported the first successful PGD case for NCL-2 involving a couple with two affected children.

Materials and methods

Family history

A couple (maternal age 28 and paternal age 34) was referred to the State Key Laboratory of Reproductive Medicine, Nanjing Medical University due to adverse birth histories of their two children. Their 3-year-old boy suffered from epilepsy, followed by regression of developmental milestones with a suspected diagnosis of NCL-2. Cytogenetic analysis showed that the maternal, paternal and proband karyotypes were normal. Independent DNA testing by exonic sequencing of TPP1, however, confirmed the presence of a maternal mutation c.824T>G (p.L275P) that was inherited by the proband. The maternal mutation was confirmed by mini-sequencing (Figure 1). No paternal mutation was identified. Assay of TPP1 from lymphocytes (Chang et al., 2012) confirmed a very low level of enzymic activity in the proband compared with the positive control, indicating the presence of a genetic defect in TPP1 of the father. On the basis of these preliminary findings, it was presumed that the father had a novel TPP1 mutation in the intronic or regulatory sequences possibly affecting TPP1 mRNA splicing or transcription. The proband’s elder sister had also suffered from same clinical manifestations as her brother and died at six years of age. After genetic counselling and written informed consent, the couple decided to undertake IVF and PGD in order to have a child without NCL-2.

Ovarian stimulation

During the stimulation cycle, pituitary desensitization was induced with daily s.c. triptorelin (Decapeptyl; Ferring), 1000 µg for the first 3 days then reduced to 500 µg per day until the day of human chorionic gonadotrophin (HCG) injection. After confirmation of down-regulation, ovarian stimulation was induced using recombinant FSH (Gonal-F; Serono) 187.5 IU administered daily. An injection of 250 mg s.c. HCG (Ovitrelle, Merck-Serono,) was given when three mature (>18 mm) follicles were observed and oocyte retrieval was performed 36 h later. Oocytes were collected under sedation using ultrasound transvaginal control.

Fertilization, embryo culture and biopsy

Oocytes were cultured in Universal IVF Medium (Origio, Denmark) for 3 h and metaphase-II eggs were fertilized by intracytoplasmic sperm injection (ICSI) using a micromanipulator system (Narishige, Japan). Each inseminated oocyte was cultured individually after ICSI in Quinn’s cleavage medium (Sage, USA) in a humidified 37°C incubator with an atmospheric environment of 5% CO2, 5% O2 and 90% N2 until day 3 after oocyte retrieval. Blastomere biopsy was undertaken using a non-contact laser (OCTAX Laser Shot-system; MTG, Germany) by making a 30-µm opening in the zona pellucida. Using a 35-µm inner diameter biopsy micropipette (Humagen, Charlottesville, NC), one blastomere was carefully extracted and placed into a drop of cleavage medium. After washing the cell carefully in phosphate-buffered saline (PBS) three times, each blastomere was placed into...
2.5 μl PBS in a sterile 0.2 ml PCR tube. Each was carefully labelled with the number of each embryo. The biopsied embryo was transferred to Quinn’s Blastocyst Medium (Sage, USA) and cultured to day 5.

PGD strategy

For the TPP1 single-gene case, this study selected the single-cell Sureplex Whole Genome Amplification Kit (BlueGnome, UK) to provide a DNA template for down-stream PCR analysis of TPP1. In previous single-gene cases for Wiskott-Aldrich syndrome and HLA matching, spinal cerebellar ataxia-3 and haemophilia A, this study group employed a preimplantation genetic haplotyping type strategy (Renwick et al., 2006, 2010) using a set of four or five STR markers located either side of the single-gene target combined with additional parental mutation analysis (Qubbaj et al., 2011) followed by preclinical testing to establish normal and affected paternal and maternal haplotypes. In these single-gene cases, Sureplex WGA on single biopsied blastomeres was able to consistently amplify a diagnostic set of STR markers, allowing the identification of unaffected embryos for transfer, even in the presence of allele drop out (ADO) of some the STR markers. Thus for the TPP1 case, this study also adopted a similar haplotyping strategy without prior ADO testing to identify a set of linked diagnostic STR markers using the UCSC database (http://genome.ucsc.edu/). The search identified seven potential STR markers (D11S1996, D11S1338, D11S1323, D11S1997, D11S1331, D11S3531 and D11S4757) within ~1 Mb of TPP1 to support the diagnosis of the maternal TPP1 mutation (Table 1). In the preclinical work up, linkage analysis performed on genomic DNA from the couple and proband showed that D11S1996 was fully informative, D11S1338, D11S1323, D11S1997 and D11S1331 were partially informative and D11S3531 and D11S4757 were non-informative (Figs. 2 and 3). Preclinical testing of three research blastomeres showed that Sureplex WGA could reliably amplify the five informative STR markers and the mutation loci with no amplification failure observed (Supplementary Figure 1, available online only), demonstrating that Sureplex WGA in combination with STR haplotyping and mutation analysis would also be a suitable strategy to make a reliable diagnosis of the patient’s embryos.

PGD

Biopsied blastomeres and PBS blank controls were therefore amplified by Sureplex WGA and embryo aneuploidy screening performed by array CGH using 24sure V3 arrays (BlueGnome) according to the standard protocol (www.24suretest.com). Resulting WGA DNA products from each embryo were diluted 1:10 in sterile water for down-stream PCR tests. Mini-sequencing (also called single-nucleotide primer extension) was performed to test the maternal mutation loci using a SNapshot Multiplex Kit (Applied Biosystems, USA) in combination with forward (5‘-GGCGGGATTGAGGCCAGTC-3′) and reverse (5‘-ACCAGCCTCATCAGGTACTGCACATCT-3′) mini-sequencing primers that bind adjacent to the maternal TPP1 mutation nucleo-
tide. Singleplex PCR reactions contained 2 μl DNA template, 0.3 μl of 5 U/μl AmpliTaq Gold DNA polymerase (Applied Biosystems), 2 μl of 20 μM primers (1 μl each of forward and reverse primer), 1 μl of 10 mM dNTPs, 5 μl of 25 mM MgCl₂, 5 μl of 10× GeneAmp PCR buffer II and 34.5 μl of sterile water to a final volume of 50 μl. PCR conditions were one cycle at 95°C for 12 min, then 15 cycles at 94°C for 30 s, 63°C for 60 s with touchdown 0.5°C per cycle and 72°C for 110 s, and finally 24 cycles at 94°C for 30 s, 56°C for 60 s and 72°C for 110 s. PCR products confirmed on agarose gels were electrophoresed on an ABI Prism 3100 Genetic Analyser (Applied Biosystems) and analysed with Genemarker software (Softgenetics, USA).

Confirmatory prenatal diagnosis

Fetus cells were sampled by amniocentesis at 18 weeks gestation and genomic DNA was extracted from the amniocytes using a QIAamp DNA micro kit (Qiagen, USA). Direct sequencing was used to confirm the presence or absence of the maternal mutation. In addition, the fetal genotype was confirmed by STR analysis using the five TPP1 linker markers. The karyotype was accessed using Focus constitutional arrays (BlueGnome) according to the standard protocol (www.cytochip.com).

Results

Following ovarian stimulation, seven oocytes were retrieved. Five of the six metaphase-II oocytes injected by ICSI fertilized and developed normally to day 3 (Table 2). One cell was biopsied from each of the five embryos and the biopsied embryos cultured further to day 5. Biopsies from embryos 2, 3, 4 and 6 were successfully amplified by Sureplex WGA, whereas the blastomere from embryo 5 failed to amplify (Supplementary Figure 2). A second blastomere was therefore removed from embryo 5 which was subsequently successfully amplified by WGA. Mini-sequencing for the maternal mutation loci and STR analysis by fluorescent PCR and allelic sizing were performed on the WGA products from the five blastomere biopsies. The maternal mutation loci (Supplementary Figure 2) and the five STR markers showed 100% amplification success with individual ADO rates of 50% (mutation loci), 20% (D11S1338), 50% (D11S1323), 20% (D11S1997), 80% (D11S1331) and 80% (D11S1996), calculated according to the STR loci shown in Figure 2 and mutation loci shown in Table 2. Despite the random pattern of ADO, reliable genotypes could be assigned for four of the five embryos (Figure 2 and Table 2). Embryos 2, 4 and 5 were diagnosed as carriers, with embryos 2 and 4 inheriting the paternal affected haplotype and the normal maternal haplotype (no maternal mutation) and embryo 5 inheriting the maternal affected haplotype and the normal paternal haplotype. Embryo 3 was homozygous affected, inheriting both affected parental haplotypes. Due to severe ADO for four of the five STR markers, the diagnosis of embryo 6 was unclear, being either a carrier or homozygous affected. Array CGH analysis revealed that the two carrier embryos 2 and 5 were euploid (Figure 4 and Table 2). In contrast, significant chromosome aneuploidies 69 XXY, complex and monosomy X were
identified in embryos 3, 4 and 6, respectively (see Supplementary Figure 3 for array CGH profiles).

On the basis of the embryonic $TPP1$ genotypes and aneuploidy results, only embryos 2 and 5 were considered for transfer. Embryo 2 was selected in preference to embryo 5 because it was subjected to only a single biopsy procedure, had developed further to the blastocyst stage and had inherited the normal maternal haplotype, confirmed by the absence of the maternal $TPP1$ mutation. Transfer of embryo 2 resulted in a positive HCG pregnancy, a single heart beat and an ongoing pregnancy. The couple agreed to have confirmatory prenatal diagnosis by amniocentesis at 18 weeks’ gestation. Mutation (Figure 1), STR marker (data not shown) and array CGH (Figure 4) analyses of amniocyte DNA showed concordance with the original embryo 2 genotype. Ultrasound scans revealed normal fetal parameters and a healthy pregnancy ensued.

Discussion

With increasing IVF success rates and advanced single-cell-based technologies, PGD is a viable alternative for couples at high genetic risk to be enabled to have a child without a serious genetic disease (Musters et al., 2010). In this study, a couple presented for genetic counselling who had two severely affected children with NCL-2, one who had since died. After considering the options of prenatal diagnosis and PGD, the couple requested PGD to avoid the potential issue of termination of pregnancy in the case of another affected child. Genetic testing identified only one of the two $TPP1$ mutations which was carried by the mother and inherited by the proband. In the absence of knowledge of the paternal $TPP1$ mutation, the PGD laboratory designed a test for the maternal mutation loci and used five STR markers linked to $TPP1$ for the primary diagnosis. In addition, array CGH was also simultaneously performed to assess the chromosomal status of the embryos. The PGD test identified embryos 2 and 5 as unaffected carriers and both were also euploid. Since embryo 2 had a higher potential for implantation as well as a safer, more defined, unaffected maternal haplotype (Table 2), it was preferentially transferred on day 5 resulting in a singleton pregnancy. Embryo 5 has been cryopreserved for a second transfer if the couple desires to have another unaffected child. Prenatal diagnosis by amniocentesis confirmed the original PGD diagnosis. The patient has a healthy ongoing pregnancy expected to go to full term.

Although the array CGH results were not pivotal to the decision as to which of two unaffected embryos to transfer, this study highlights the potential value of performing single-gene PGD in conjunction with aneuploidy screening, regardless of maternal age. Importantly, no additional damage was done to the embryos in determination of their aneuploidy status. The strategy relied heavily on the single-blastomere WGA reaction to generate not only amplified sequences surrounding $TPP1$ but also target sequences across the genome compatible with the probes on the array. In other previous single-gene cases conducted for Wiskott-Aldrich syndrome and HLA matching, spinal cerebellar ataxia-3 and haemophilia A using Sureplex WGA, the target single-gene sequences in the biopsied blastomeres were adequately covered by WGA, allowing a reliable PCR diagnosis using a STR haplotyping and mutation analysis-based approach (data not shown). These preliminary findings from the three previous single-gene cases and the $TPP1$ case reported here suggest that Sureplex WGA may be generally applicable for single-gene PGD cases in conjunction with aneuploidy screening. Successful single-gene PGD combined with aneuploidy screening has also been previously reported for Von Hippel–Lindau disease (Obradors et al., 2009) and Lynch syndrome (Daina et al., 2012) using the multiple displacement amplification-based WGA system Genomiphi, with aneuploidy screening performed using a short metaphase CGH protocol. In a recent single-cell study where the commercial WGA systems Genomiphi, Genomeplex and Repli-g were all compared using genome-wide SNP analysis...
genotyping coverage was 74%, 78% and 88% and ADO rates 14%, 11% and 4%, respectively, suggesting that other types of WGA systems may also be used for combined single-gene and aneuploidy screening. The application of deep sequencing as described by Treff et al. (2013) to all current single-cell WGA systems will ultimately provide more

Figure 3 Genescan profiles of short tandem repeat D11S1338 for parents, proband and embryo 2. The true allelic peaks in blue (six FAM-labelled PCR products) are marked by the grey shading. The other unmarked blue peaks to the left represent PCR stutter bands of the (CA)_n repeat within the short tandem repeat sequence. Embryo 2 inherited the affected allele from the father and normal allele from the mother.

Table 2 Embryo morphology and final genotypes.

<table>
<thead>
<tr>
<th>Embryo ID</th>
<th>Day-3 morphology</th>
<th>Day-5 morphology</th>
<th>Paternal haplotype</th>
<th>Maternal haplotype</th>
<th>Maternal mutation</th>
<th>Aneuploidy screening</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>7 cells</td>
<td>Expanded blastocyst</td>
<td>Affected</td>
<td>Unaffected</td>
<td>Not found</td>
<td>46, XX</td>
<td>Euploid, carrier^a</td>
</tr>
<tr>
<td>3</td>
<td>8 cells</td>
<td>Expanded blastocyst</td>
<td>Affected</td>
<td>Affected</td>
<td>Not found^b</td>
<td>69, XXY</td>
<td>Aneuploid, affected</td>
</tr>
<tr>
<td>4</td>
<td>8 cells</td>
<td>Arrested</td>
<td>Affected</td>
<td>Unaffected</td>
<td>Not found</td>
<td>Complex</td>
<td>Aneuploid, carrier</td>
</tr>
<tr>
<td>5</td>
<td>8 cells</td>
<td>Morulae</td>
<td>Unaffected</td>
<td>Affected</td>
<td>Found</td>
<td>46, XX</td>
<td>Euploid, carrier</td>
</tr>
<tr>
<td>6</td>
<td>10 cells</td>
<td>Early blastocyst</td>
<td>Affected</td>
<td>Uncertain^b</td>
<td>Not found</td>
<td>45, XO</td>
<td>Aneuploid, carrier?</td>
</tr>
</tbody>
</table>

^aTransferred and ongoing pregnancy.
^bAllele drop out.
^cKaryotype = trisomy for chromosomes 1, 2, 5, 6, 11, 13, 14, 16, 17, 19, disomy for X, monosomy for Y and nullisomy for chromosomes 10 and 12.

(Treff et al., 2011b), genotyping coverage was 74%, 78% and 88% and ADO rates 14%, 11% and 4%, respectively, suggesting that other types of WGA systems may also be used for combined single-gene and aneuploidy screening. The application of deep sequencing as described by Treff et al. (2013) to all current single-cell WGA systems will ultimately provide more
comprehensive information about the suitability of each WGA system for best practice single-gene PGD.

More recently, SNP arrays in conjunction with WGA also hold promise for simultaneous single-gene and chromosome screening (Handyside et al., 2010) as well as translocation testing (Treff et al., 2011a). Similar to fluorescent PCR and STR selection, analysis of the genomic DNA family samples is also a necessary prerequisite to the clinical case to establish SNP linkage to maternal, paternal and proband haplotypes. However, SNP arrays offer the advantage of a one platform technology with less processing of samples, lower labour costs and sophisticated SNP analysis software to make an automated diagnosis. In addition, the consequences of ADO on SNP arrays are not so detrimental since only multiple heterozygous SNP (no ADO) are used to make the final diagnosis. Detailed validation studies are continuing and the results so far suggest that this technology may eventually supersede all current technologies for the majority of PGD cases in the future.

Although the strategy applied for the TPP1 case met with success in the first PGD cycle, the major deficiency was performing PGS at the cleavage-stage of embryo development. It is well known that cleavage-stage embryos exhibit high rates of mosaicism (van Echten-Arends et al., 2011) and well-designed RCTs using array CGH or SNP-based arrays to determine clinical benefit are urgently needed. In the case at hand, it was possible that the transferred embryo 2 was a diploid/aneuploid mosaic, even though the cell actually biopsied was euploid. Given this possibility, embryo 2 may therefore have failed to implant due to lack of viability and developmental competence, leading to a failed PGD case. Nonetheless, the molecular strategy can easily be translated to the blastocyst stage in future single-gene PGD cases where biopsied trophectoderm cells known to be representative of the inner cell mass (Fragouli and Wells, 2012) exhibit lower levels of aneuploidy (Fragouli and Wells, 2012; Fragouli et al., 2008) than cleavage-stage embryos, and ADO rates are substantially lower due to biopsy and testing of multiple trophectoderm cells. Recent studies of PGS at the blastocyst stage using array CGH or SNP arrays report higher pregnancy rates (Treff, 2012). Further, a randomized controlled trial of good morphological grade blastocysts and single-embryo transfers showed significantly higher implantation and pregnancy rates when euploid embryos were preselected by array CGH (Yang et al., 2012). These findings are very encouraging, suggesting that any molecular technologies used for simultaneous single-gene PGD and chromosome screening should now be applied to blastocysts rather than cleavage-stage embryos to improve outcomes for PGD patients.

In conclusion, to improve the overall success rate of single-gene PGD and bearing in mind the interests of the patient who pays a high cost for each PGD cycle, PGD laboratories with modern molecular diagnostic technologies and robust blastocyst culture and biopsy capabilities should now consider offering single-gene PGD in conjunction with total aneuploidy testing. By this approach, failed PGD cycles involving the transfer of unaffected but chromosomally abnormal embryos will be eliminated, maximizing the chances for patients carrying a single-gene defect to achieve a disease-free ongoing pregnancy that will hopefully develop to full term.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.rbmo.2013.04.011.

References


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