Reproductive BioMedicine Online (2015) ■■, ■■–■■

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ARTICLE

Karyomapping allows preimplantation genetic diagnosis of a de-novo deletion undetectable using conventional PGD technology

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Carles Giménez obtained his Bachelor's degree in Biology in 1989 at Universitat Autònoma de Barcelona (UAB, Catalonia). He started his research work focused on the development of PGD techniques, and gained his PhD in Biological Sciences from UAB in 1995. He is also a Senior Embryologist (ESHRE, 2008), Genetic Counsellor (Pompeu Fabra University, 2009) and accredited geneticist (AEGH, 2015). He is one of the PGD/PGS pioneers, and was involved in the teams that achieved the first pregnancies to term after PGD in Spain. In 2003 he cofounded Reprogenetics in Barcelona. Since then, he has been the molecular laboratory director and the scientific co-director.

Abstract Preimplantation genetic diagnosis (PGD) was carried out for a couple carrying a de-novo deletion in the *TSC2* gene, responsible for tuberous sclerosis. Karyomapping, a method employing genome-wide analysis of single nucleotide polymorphisms (SNP), was used as PGD protocol. Analysis of DNA from the affected parent using karyomapping confirmed the region covered by the deletion and revealed more than 30 SNP located within the affected region. These SNP were subsequently used for embryo diagnosis (deletion revealed by hemizygosity and/or reduced probe intensity). Seven blastocyst embryos underwent trophectoderm biopsy followed by vitrification. Biopsied cells were subjected to comprehensive aneuploidy screening using microarray comparative genomic hybridization (aCGH), with karyomapping for the detection of embryos carrying the mutant *TSC2* gene carried out in tandem. Two embryo transfers were performed, the second of which resulted in the birth of a child. This study highlights that karyomapping may be applicable to a subset of de-novo mutations undetectable using standard PGD strategies. Additionally, karyomapping results were in complete concordance with aCGH, both methods revealing the same aneuploidies in the embryos tested. It was concluded that karyomapping may represent a valuable advance in cases of PGD for monogenic diseases.

http://dx.doi.org/10.1016/j.rbmo.2015.08.017
1472-6483/

Please cite this article in press as: Carles Giménez, et al., Karyomapping allows preimplantation genetic diagnosis of a de-novo deletion undetectable using conventional PGD technology, Reproductive BioMedicine Online (2015), doi: 10.1016/j.rbmo.2015.08.017

KEYWORDS: karyomapping, preimplantation genetic diagnosis, single gene diseases, single nucleotide polymorphism, tuberous sclerosis, whole genome amplification

Introduction

Tuberous sclerosis (TSC) (OMIM: 191100) is a multi-system disorder with a dominant mode of inheritance, characterized by hamartomas in the brain, skin, eyes, heart, lungs and kidneys (Yates, 2006). These tumours are usually benign, but they are associated with significant morbidity and mortality. Of most clinical consequence are central nervous system tumours and renal disease. The disease is caused by mutations in two tumour-suppressor genes: *TSC1* (9q34, OMIM 605284) and *TSC2* (16p13, OMIM 191092).

The diagnosis of TSC is usually based on clinical and radiological findings. Mutations can be identified in around 85% of individuals who meet diagnostic criteria for TSC, with the majority (69%) found in *TSC2*. Approximately two-thirds of affected individuals have TSC as the result of a de-novo mutation (http://www.ncbi.nlm.nih.gov/books/NBK1220/).

Preimplantation genetic diagnosis (PGD) is an alternative to prenatal testing that enables people with a specific inherited condition in their family to avoid passing it on to their children. It involves the creation of several embryos using assisted reproductive technology, followed by the removal of one or more cells and detection of the mutant gene. Embryos found to be unaffected can be transferred to the mother's uterus whereas those with the mutation are discarded. Using PGD, high-risk patients can avoid issues of pregnancy termination or the birth of an affected child.

The detection of a single gene disorder (SGD), such as TSC, in cells biopsied from preimplantation embryos remains challenging because sensitive multiplex-polymerase chain reaction (PCR) methodologies are required in order to amplify specific DNA fragments (e.g. the mutation site and/or linked polymorphisms) to detectable concentrations. Problems such as allele drop out (ADO – the failure to amplify one of the two parental alleles in the biopsied cell) and contamination with extraneous DNA are important sources of misdiagnosis. To reduce the impact of these difficulties, current standard practice guidelines recommend combining amplification and analysis of several closely linked polymorphisms, along with (when possible) direct mutation detection (Harton et al., 2011; The Preimplantation Genetic Diagnosis International Society, 2008). Identification of informative polymorphisms and design and optimization of a multiplex-PCR capable of amplifying all of the necessary loci from a single cell, requires a significant amount of laboratory work. This usually leads to a delay in treatment while the protocol is created and validated and adds considerably to the cost of PGD.

Recently, karyomapping has been proposed as an alternative to conventional PCR-based protocols (Handyside et al., 2010; Natesan et al., 2014). Essentially, karyomapping involves genome-wide linkage analysis, in which several hundred thousand single nucleotide polymorphisms (SNP) scattered throughout the genome are genotyped in the two parents and their embryos. Each chromosomal region has a unique SNP fingerprint, allowing the inheritance of chromosomal segments (and the genes they contain) to be tracked from one generation to the next. By comparing SNP results obtained from the parents to those obtained from other family members of known genetic status (e.g. another relative carrying the same mutation as one of the parents) the combination of SNP alleles associated with a chromosome carrying a mutant gene can be identified. Transfer of embryos carrying this chromosome (or SNP pattern) can then be avoided.

The main advantage of karyomapping is that it can be used for diagnosis of any familial SGD without the need to develop a patient-specific test, greatly reducing the time required for work-up prior to PGD. Moreover, the detection of individual parental chromosomes using SNP allows trisomies of meiotic origin and monosomies to be revealed. Testing for aneuploidy in the embryos of patients undergoing IVF treatment, with transfer of those found to be chromosomally normal, has been shown to yield an improvement in ongoing pregnancy rates and is also anticipated to reduce risks of miscarriage and Down syndrome (Forman et al., 2013; Scott et al., 2013; Yang et al., 2012). Thus, the combination of SGD diagnosis together with aneuploidy screening may improve the clinical outcome of PGD cases.

Materials and methods

Patient information – genetic and clinical

A 32-year-old woman diagnosed as affected with tuberous sclerosis type 2, with renal symptoms and one year of secondary infertility, was referred to us to undergo an IVF–PGD cycle. Hysterosalpingography showed a non-permeable Fallopian tube. Her 33-year-old husband presented normal spermiogram and fluorescence in-situ hybridization (FISH) sperm test. Both had normal karyotypes.

Using multiplex ligation-dependent probe amplification (MLPA) the woman was shown to be a carrier of a de-novo deletion encompassing exons 1 to 15 and also the 5′ region of the *TSC2* gene.

Preliminary work-up

The patients received appropriate counselling and informed consent was obtained for all procedures undertaken. The clinics and laboratories involved in providing treatment hold all licences required to permit IVF and PGD to be offered. Since PGD is considered to be a standard treatment, which requires patient-specific optimization and the development of novel protocols as a matter of routine, no further ethical approval was sought on this occasion. Traditional PGD work-ups for patients at risk of transmitting a single gene disorder require a high degree of customization, comprising the design and optimization of PCR primers for amplification of the mutation site and several linked short tandem repeat (STR) polymorphisms. However, in this case, a de-novo mutation was responsible for the disorder. Since no other family members carried the mutation, linkage analysis was not possible. Direct

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Table 1 List of single nucleotide polymorphisms (SNP) used as indirect detection of the mutation.

SNP	*Chr. Location*	*Heterozygosity %*
rs45517102 G/A	16:2103454	Unknown
rs45505995 T/G	16:2103455	Unknown
rs45468592 A/G	16:2106195	Unknown
rs45517117 G/A	16:2106196	Unknown
rs45460895 G/A	16:2105521	Unknown
rs45484992 T/G	16:2105522	Unknown
rs75752552 C/T	16:9920954	50
rs75051685 G/T	16:9920974	50
rs63820802 C/A	16:2100009	50
rs2238374 T/A	16:2102486	50
rs28502796 A/G	16:2102611	48.2
rs111700202 G/A	16:2106521	50
rs112305151 G/T	16:2106610	50
rs78160478 G/T	16:2107322	11.1
rs112577215 C/T	16:2107421	0.3
rs60154230 C/G	16:2107455	1.8
rs113296145 G/A	16:2108963	50
rs113419658 G/A	16:2109019	50
rs2516740 T/G	16:9720110	37.3
rs2516739 C/T	16:9720158	37
rs114480195 G/A	16:9920181	2.3
rs3760042 A/G	16:9920232	50
rs111495630 C/T	16:2104787	2
rs78488281 T/C	16:2104793	5.2
rs113328694 C/T	16:2105036	2.1
rs7320636 A/G	16:2105055	41.5

All primer sequences and detailed PCR conditions are available upon request.

mutation detection was also precluded due to the fact that the genetic lesion was not a simple change in DNA sequence (which could be amplified by PCR and then detected) but a large deletion. MLPA had suggested the deletion to be at least 16.9 kb in size, but the breakpoints had not been precisely defined and, consequently, it was impossible to design PCR primers capable of revealing the mutation.

Initially, an attempt was made to find informative SNP inside the deleted region (**Table 1**). An unaffected embryo would show alleles inherited from both parents, while an affected embryo would show homozygosity (hemizygosity) for all polymorphisms within the deleted region, with all detected alleles derived from the unaffected parent. Despite investigating multiple polymorphisms, none were found to have genotypes appropriate for accurate determination of inheritance from the parents.

IVF and embryo biopsy

The couple underwent two cycles of intrauterine insemination (IUI) and a regular IVF cycle without PGD, with fresh and cryopreserved embryo transfers, all with negative pregnancy results. A new strategy of IVF–PGD utilizing a combination of karyomapping and microarray comparative genomic hybridization (aCGH) was proposed and the patients gave informed consent for this treatment. The family received counselling for PGD at Reprogenetics in Barcelona. Reprogenetics laboratories in Barcelona and Oxford carried out aCGH and karyomapping, respectively.

Following an antagonist ovarian stimulation protocol, IVF–PGD was performed. After human chorionic gonadotrophin (HCG) trigger, transvaginal ultrasound-guided oocyte retrieval was carried out. Fertilization involved ICSI and the resulting embryos were kept in culture up to the blastocyst stage. Cells were sampled from the embryos on day 5 of development. Briefly, blastocysts were biopsied by drilling a hole through the zona pellucida with a laser (ZILOS-tk, Hamilton Thorne, Inc., Beverly, MA, USA). Suction was applied to the trophectoderm with a biopsy pipette followed by cutting a small piece of tissue (2–4 cells) using the laser. The biopsied cells were washed and collected into sterile PCR tubes. After trophectoderm biopsy embryos were vitrified (Cryotop-B, Kitazato BioPharma, Fuji, Japan) for future transfer.

aCGH

Samples were lysed and the DNA released was fragmented and subjected to whole-genome amplification with the Repli-g® single cell kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Two reference DNA samples (genomic male and female DNA) and one negative control followed the same amplification protocol as the embryo samples. Electrophoresis was performed to confirm that all samples and the positive controls amplified appropriately. The negative control did not produce any amplified DNA, indicating that there was no contamination. Amplified embryo DNA samples and references were labelled with Cy3 and Cy5 using the Illumina fluorescent labelling system, according to the manufacturer's instructions. Labelled samples and reference DNA were combined and applied to a 24Sure microarray (Illumina, USA) and co-hybridized overnight. After hybridization the slides were washed as described by Jaroudi and Wells (2013), dried by centrifugation and scanned with a microarray scanner (InnoScan 710AL, Innopsys, Carbonne, France). Scanned images were analysed and quantified, and whole chromosomal copy number ratios were reported using the CytoChip algorithm fixed settings in BlueFuse Multi Software, version 3.3 (Illumina).

Karyomapping

Karyomapping involved the use of the Infinium HumanKaryomap-12 DNA Analysis Kit, (Illumina). The protocol essentially followed that described by Natesan et al. (2014). Genomic DNA samples from the couple were tested using a HumanKaryomap-12 BeadChip (Illumina) and analysed using BlueFuse Multi version 4.1 software (Illumina). This allowed genotyping of SNP within and flanking the *TSC2* gene, including the region affected by the deletion. It was also possible to obtain quantitative (copy number) information by considering the amount of DNA hybridized to probes mapping to within the gene. Aliquots of the same whole-genome amplified (MDA) samples from embryos, used for aCGH, were also analysed using the karyomapping protocol. A DNA sample from

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the affected patient's mother was also available. This was also subjected to karyomapping analysis in order to allow phasing of SNP alleles (i.e. revealing which of the patient's two copies of chromosome 16 each SNP allele was situated upon).

Results

Development of a customized conventional PCR protocol

Twenty-six SNP within the deleted region and nine STR in the vicinity of the affected gene were assessed in an effort to detect the chromosome carrying the mutant copy of *TSC2*. One STR was found to be fully informative (both parents heterozygous and sharing no alleles in common) and five more were partially informative (the parents having three alleles between them). However, the use of these polymorphisms to deduce a disease-associated haplotype that could be used for diagnostic purposes was precluded by the de-novo nature of the mutation. Although closely linked to *TSC2*, none of these STR lay within the deleted region of the gene and consequently they could not be used for direct mutation detection.

Regarding SNP analysis, five of them were found to be partially informative, that is, one of the paternal alleles had the same base as the maternal healthy allele (e.g. unaffected father's genotype A B; affected mother's genotype A –; potential unaffected embryo genotypes A A, A B; affected embryo genotypes A –, B –; the A – and A A genotypes are indistinguishable using standard qualitative genotyping methods). Because of this limitation, half of the healthy embryos would have to be discarded due to uncertainty over their disease status. A genetic counselling session was scheduled with the patients to explain both the poor informativity found after the analysis of 26 SNP and the impossibility to correctly diagnose half of their healthy embryos. After this, the couple opted to reject PGD, using the conventional methodology.

IVF–PGD cycle using karyomapping

Preliminary analysis of DNA samples from the parents indicated that karyomapping technology might allow the *TSC2* deletion to be detected. After further counselling, the couple opted to proceed with PGD using a karyomapping protocol.

A total of 32 SNP assessed by the HumanKaryomap-12 BeadChip were found to be within the deleted area of the gene and the mutation was clearly visible in the genomic DNA of the affected patient, appearing as an area devoid of any heterozygous SNP (hemizygosity for all loci). Of note, only one of the 32 SNP had been assessed during the earlier efforts to devise a conventional PGD protocol. Embryos with two normal copies of *TSC2* usually displayed more than 10 heterozygous SNP within the deleted region, whereas each of the embryos carrying the mutation had just one site of heterozygosity (presumably the result of a technical problem affecting the associated probe). A quantitative difference in the amount of DNA annealed to the SNP probes within the deleted region was also apparent (**Figure 1**).

After ovarian stimulation, 23 oocytes were obtained. ICSI was performed for 21 metaphase II oocytes resulting in 16 2PN/2PB zygotes. During embryonic development, eight embryos type A (best morphology), two type B, four type D and two arrested were observed on day 2; two embryos type A, six type B, two type D and four arrested were seen on day 3; and four embryos type B (BiE BB, BiE AB, BE AB, BE AB), one type C (BC) and two type D (BT, BC) were available on day 5 (ASEBIR, 2010 evaluation criteria). Trophectoderm biopsy was performed on all the blastocysts obtained, followed by vitrification (Cryotop-B, Kitazato BioPharma).

Because karyomapping has not yet been fully validated for the purpose of aneuploidy screening, aCGH was employed to detect chromosome copy number abnormalities followed by karyomapping to assess inheritance of the *TSC2* mutation. Trophectoderm samples were successfully amplified from all of the embryos available on day 5. Microarray CGH analysis showed that one embryo (#11) had a monosomy affecting chromosome 6, while all of the other embryos were found to be euploid. All seven embryos were then analysed by karyomapping (**Table 2**). Karyomapping confirmed that embryo 11 had a loss of chromosome 6 material and detected no aneuploidies in any of the other embryos. Three of the chromosomally normal embryos were shown to carry the *TSC2* deletion, while the other three were unaffected (**Figure 1**). One unaffected euploid embryo was warmed and transferred to the uterus but no pregnancy was established. One month later the remaining two unaffected euploid embryos where warmed and transferred, resulting in a single ongoing pregnancy, followed by the birth of a child.

Discussion

An attempt was made to create a classical PGD test for a patient with a de-novo deletion (estimated at 16.9 kb), removing several exons of the *TSC2* gene. After assessing a total of 26 SNP within the deleted region, the protocol developed was still insufficient for the conclusive diagnosis of all potential embryos produced by the couple. For this reason the patients chose to reject PGD and tried to conceive naturally. Had yet more SNP within the deleted region of the gene been tested, it is likely that a conventional PGD protocol, able to reliably distinguish unaffected embryos from those carrying the mutation, would eventually have been developed. However, the amount of time and the costs associated with a prolonged search for informative SNP was prohibitive, leading to the abandonment of attempts to create a traditional PGD test.

It was considered that a new PGD methodology, karyomapping (Handyside et al., 2010; Natesan et al., 2014), might overcome the difficulties in identifying informative polymorphisms within the deleted region. The protocol allows simultaneous analysis of more than 300,000 SNP across the whole genome, including 630 within a region encompassing the *TSC2* gene and 2 Mb either side. Most karyomapping protocols work by considering the genotype of SNP alleles in close proximity to a familial mutation, revealing the series of contiguous alleles (known as a haplotype) associated with the affected chromosome. The inheritance of the mutant-linked haplotype can be traced from the affected parent(s) to their

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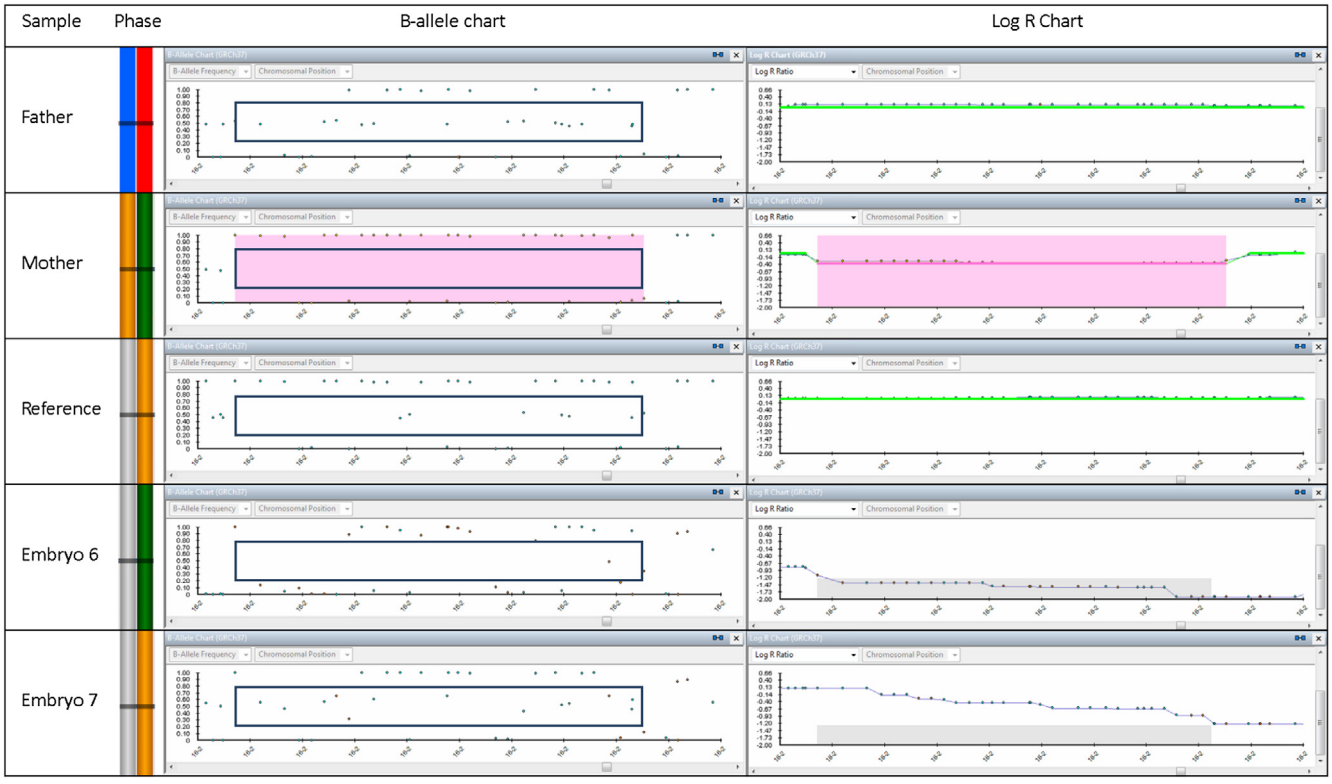

Figure 1 Detailed B-allele and Log R charts in the deleted region (in pink) of the *TSC2* gene after karyomapping analysis. Region displayed is 270 Kb in size (16:1880000–2150000). The maternal haplotypes are shown in yellow and green. The paternal haplotype inherited is not shown. The mother and embryo 6 have the deletion, determined by homozygosity for single nucleotide polymorphisms (SNP) in the critical region (heterozygous SNP in the deleted area are indicated by spots within the box in the B-allele chart for each embryo). The Log R Chart confirms reduced quantities of DNA bound to probes within the deleted region, indicative of reduced copy number (probes fall into the grey shaded box representing low fluorescence values). All embryos carrying the deletion inherited the same maternal chromosome 16 haplotype (green). Unaffected blastocysts (e.g. embryo 7 shown here) display heterozygous SNP within the deleted area (B-allele), higher concentrations of DNA hybridized to probes (Log R), and the opposite maternal haplotype (yellow) to affected embryos.

embryos, thus allowing a diagnosis to be made. However, in this specific case the *TSC2* mutation was *de novo*, precluding the use of a standard linkage-based karyomapping strategy (it is not possible to determine which SNP alleles are associated with the mutation unless other family members of known mutation carrier status are available for comparison). Nonetheless, analysis of DNA from the affected mother suggested that SNP data produced using the karyomapping protocol might allow direct detection of affected embryos. Results from the mother indicated that the mutation could be visualized as an area of *TSC2* lacking any heterozygous SNP (hemizygosity due to loss of one copy of the gene). Additionally, there was a detectable reduction in the quantity of DNA hybridized to SNP probes within the region covered by the

Table 2 Results of the embryo analysis using karyomapping and aCGH.

Embryo #	*aCGH results*	*Karyomapping results*		*Final diagnosis/recommendation*
		Deletion status	*Chr. abnorm.*	
6	Euploid	Carrier	–	Affected by tuberous sclerosis and euploid/not recommended for embryo transfer
7	Euploid	Normal	–	Not carrier and euploid/this embryo could be considered for transfer
8	Euploid	Normal	–	Not carrier and euploid/this embryo could be considered for transfer
10	Euploid	Carrier	–	Affected by tuberous sclerosis and euploid/not recommended for embryo transfer
11	Aneu	Normal	–6	Not carrier and aneuploid/not recommended for embryo transfer
12	Euploid	Normal	–	Not carrier and euploid/this embryo could be considered for transfer
20	Euploid	Carrier	–	Affected by tuberous sclerosis and euploid/not recommended for embryo transfer

aCGH = microarray comparative genomic hybridization; Aneu = aneuploid; Chr abnorm = chromosome abnormality.

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deletion. It was considered that the combination of qualitative and quantitative data obtained by this approach provided an excellent opportunity to detect affected embryos.

This diagnostic strategy also has the potential to reveal the majority of aneuploidies affecting human preimplantation embryos. However, since karyomapping has not yet been fully validated for this purpose, it was decided that aCGH would be used in parallel, in order to provide information concerning chromosomal status. It was hoped that the identification and exclusion of aneuploid embryos would improve the chances of transferring a viable embryo and reduce the risks of miscarriage and Down syndrome.

Ultimately, the karyomapping protocol worked well, successfully identifying the deletion in the affected embryos tested and confirming its actual size to be 210 kb, significantly larger than originally thought. All of the embryos carrying the mutation displayed a deficiency of heterozygous SNP in the affected region of the gene (**Figure 1**; B-allele chart). Reduced quantities of DNA bound to probes within the deleted part of *TSC2* were also apparent in the majority of cases (**Figure 1**; Log-R chart). Finally, karyomapping confirmed that all embryos considered to be affected shared an identical maternal haplotype in the vicinity of *TSC2* (i.e. they had inherited the same maternal copy of chromosome 16; **Figure 1**, Phase). Analysis of chromosome copy number using karyomapping revealed that chromosome 6 was abnormal in one of the embryos, a defect which was also identified by parallel aCGH analysis. The detection of aneuploidy in a blastocyst from a young, fertile couple emphasizes the fact that potentially lethal chromosome abnormalities occur even in the embryos of good prognosis patients.

This study confirmed that karyomapping is a powerful and versatile new approach for the diagnosis of single gene disorders in preimplantation embryos, with the possibility to go beyond linkage analysis and provide direct detection of mutations, some of which may be refractory to conventional PGD methods. The potential for karyomapping to provide a simultaneous identification of aneuploidy was also confirmed. While the cost of consumables needed for karyomapping is significantly higher than that of the reagents required for conventional PGD methods, the amount of time spent designing, optimizing and validating the protocol for each family is much less, and consequently the overall cost of the two strategies ends up being similar. These results support the argument that the huge reduction in work-up times achieved using karyomapping is just one of several significant technological benefits of the method and that some patients will also benefit from aneuploidy screening and/or the detection of challenging mutations.

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Declaration: The authors report no financial or commercial conflicts of interest.

Received 1 April 2015; refereed 29 June 2015; accepted 20 August 2015.