Case report: birth of healthy twins after preimplantation genetic diagnosis of propionic acidemia

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Abstract

Purpose Development of an *ad hoc* protocol for the preimplantation genetic diagnosis of propionic acidemia in a couple carrying the mutations c.737G>T (G246V) and c.1218del14ins12 (ins/del) in the *PCCB* gene. Propionic acidemia is an autosomal recessive metabolic disorder where the body is unable to process certain parts of proteins and lipids. Symptoms manifest few days after birth and sometimes progress to more serious medical problems, including heart abnormalities, coma and death.

Methods Four short tandem repeat markers closely linked to the *PCCB* gene were tested, in order to support the direct mutation detection diagnosis. Multiplex fluorescent hemi-nested polymerase chain reaction followed by fragment analysis and minisequencing was used.

Capsule A description of a PGD cycle for the detection of propionic acidemia, resulting in the birth of two healthy boys.

A couple with a dead son affected of propionic acidemia underwent preimplantation genetic diagnosis to prevent the transmission of this disease to their offspring

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Results Fourteen single blastomeres from nine embryos were tested and two carrier embryos were transferred, resulting in the birth of two healthy boys.

Conclusions Preimplantation genetic diagnosis represents a valid reproductive option for couples affected of propionic acidemia, in order to avoid transmission to offspring.

Keywords Molecular PGD · Monogenic disease · Preimplantation genetic diagnosis · Propionic acidemia

Introduction

In 1968, Gardner and Edwards succeeded in sexing rabbit embryos at approximately day 5 after fertilization [1]. Since then, the rapid development of the genetic and assisted reproductive techniques permitted the birth of the first baby after in vitro fertilization (IVF) in 1978 and the first successful application of Preimplantation Genetic Diagnosis (PGD) in 1990 [2]. PGD has been developed for couples at high risk of transmitting a genetic disease to their offspring. It is performed on single cells biopsied from in vitro fertilised embryos at the third day of culture (6–8 cells stage). Only healthy embryos are transferred to the uterus. Therefore, PGD represents an alternative to prenatal diagnosis and termination of pregnancy in case of affected foetus. Hence, it has been made available for a large number of rare genetic disorders [3] and the number of cycles increases year by year [4]. In this framework, we describe for the first time a novel protocol for the preimplantation genetic diagnosis of propionic acidemia.

Propionic acidemia (PA) (OMIM #606054) also known as propionic aciduria, propionyl-CoA carboxylase deficiency or ketotic glycinemia, is an autosomal recessive metabolic

disorder, classified as a branched-chain organic acidemia [5]. In most cases, the first symptoms of PA appear shortly after birth. Clinical features include metabolic acidosis, ketosis, vomiting and lethargy. These symptoms sometimes progress to more serious medical problems, including heart abnormalities, seizures, coma, and possibly death. Less commonly, the signs and symptoms of PA appear during childhood and may come and go over time. Some affected children experience intellectual disability or delayed development. In children with this late-onset form of the condition, episodes of more serious health problems can be triggered by prolonged periods without food (fasting), fever, or infections [6]. The worldwide incidence of PA is very low (1:50,000) and varies ranging from 1:17,400 in Japan [7] to 1:250,000 in Germany [8]. In specific populations as the Greenland Inuit, the incidence is much higher (1:1,000) due to a founder effect and genetic drift [5]. In Spain, it is difficult to determine the real incidence because there is not a neonatal screening established in the Spanish territory. In addition, in many cases, there exists a neonatal demise without diagnosis. Nowadays, there are only data available for Galicia, where the incidence is 1:106,662 [9].

Etiologically, propionic acidemia is caused by a deficiency of the mitochondrial protein propionyl-CoA carboxylase (PCC), one of the four biotin-dependent enzymes. PCC is a multimeric protein composed of two different α - and β -PCC subunits, nuclearly encoded by the *PCCA* and *PCCB* genes, respectively [10]. Mutations in either the *PCCA* or *PCCB* genes can cause reduced or deficient enzyme activity. In both genes, missense mutations are the most frequent defects (39 and 46% for *PCCA* and *PCCB* respectively), followed by small insertions/deletions and splicing mutations (24–29% each in either gene), most resulting in a truncated protein.

The purpose of this paper is to show, for the first time, the successful application of PGD for propionic acidemia, resulting in the birth of two healthy boys.

Case report

The couple comprised by a 33 year old woman and a 32 year old man attended genetic counselling after their 2 month old baby died. The baby was clinically diagnosed with propionic acidemia by determining the enzymatic activity of propionyl-CoA carboxylase. The couple referred no more affected members in their families and also reported a genetic study of his dead son where the two propionic acidemia causating mutations had been identified. The baby was a compound heterozygote for c.737G>T (G246V) (exon 7) and c.1218del14ins12 (ins/del) (exon 12) mutations in the *PCCB* gene, confirming the clinical diagnosis. The parents of the affected child, as in an

autosomal recessive disease, are obligate asymptomatic carriers. In this case, the parents did not have any genetic study. The genetic risk, the success rates, the risk of misdiagnosis and the importance of prenatal diagnosis in case of pregnancy were specifically discussed with the couple. The complete PGD process was explained to the patients and their queries were answered. The couple signed an informed consent form for PGD testing.

Materials and methods

Blood samples in EDTA were requested from both members of the couple and DNA sample from the dead baby was provided. DNA extraction from blood was carried out following the protocol described in the High pure PCR template preparation kit (Roche, Switzerland). Mutation analysis was first performed on 10–30 ng of genomic DNA in a single PCR to confirm the mutations and to determine the informative short tandem repeat (STR) markers, then on 1 ng of diluted genomic DNA in a heminested multiplex PCR with the informative STR markers and finally on single lymphocytes collected from both parents with the same PCR conditions that worked for 1 ng of genomic DNA. Oligonucleotides were tested in order to discard possible human DNA contamination.

Preclinical studies and lymphocyte validation was performed prior to the in vitro fertilization (IVF) cycle, following recommended international guidelines on PGD (3, 10) and the norm UNE-EN ISO 9001:2000 [11, 12]. Four STR markers closely linked to the *PCCB* gene were tested. The molecular analysis protocol was the same for lymphocytes (preclinical set-up) and blastomeres (clinical PGD cycle). Single cells were isolated and lysed as previously described [13]. PCR amplification was performed using the outer and inner oligonucleotide primers listed in Table 1. First round multiplex PCR containing the external primers was carried out in a total volume of 25 μ L containing 200 μ M dNTP, 1.5 mM $MgCl_2$, 1X PCR buffer, 1 unit *AmpliTaq Gold* polymerase (Applied Biosystems, U.S.A) and 0.4 μ M each of the outer forward and reverse primers. One microlitre from the first round PCR was used as template for separate second round PCR reactions with fluorescently labelled inner forward and reverse primers in a total volume of 10 μ L. PCR reactions were undertaken using a 9700 PCR system (Applied Biosystems, U.S.A.). Twenty five thermal cycles were performed for the outer primers and 50 for the inner primers at 95°C for 1 min, 50°C for 45 s, and 72°C for 1 min. The cycling was preceded by a 5 min initial denaturation step at 95°C and ended with an additional elongation step of 7 min at 72°C. Amplified inner products were electrophoresed in an automated genetic analyser 3730xl (Applied Biosystems, USA). Results were

Table 1 Primer sequences used for preimplantation genetic diagnosis of propionic acidemia

	Name	Heterozygosity	Gene distance (Mb)	Primer Sequences
<i>Fout</i> forward outer primer, <i>Fin</i> forward inner primer, <i>R</i> reverse primer, <i>SEQ</i> minisequencing, <i>N/A</i> not applicable, <i>uk</i> unknown ^a Rosen, N., V. Chalifa-Caspi, O. Shmueli, A. Adato, M. Lapidot, J. Stampnitzky, M. Safran, and D. Lancet (2003). GeneLoc: Exon-based integration of human genome maps. Bioinformatics 19(S1). URL: http://genecards.weizmann.ac.il/geneloc	PCCB-E7	N/A	N/A	<i>Fout</i> : 5' CATCTTGGCTGAATCAACTC 3' <i>R</i> : 5' CAACAAGGTCATCTTCAAGG 3' <i>Fin</i> : 5' 6FAM-CTCTGAGGTTGACTGTTCTGG 3' <i>SEQ</i> : 5' TGGTGGTGTGGGTCTTGGCA 3'
	PCCB-E12	N/A	N/A	<i>Fout</i> : 5' GGCTGTGTAAGGAATGGC 3' <i>R</i> : 5' CTCCTGGTGATGACTGTGAC 3' <i>Fin</i> : 5' NED-CTGAGAGTGGCAGGATAACC 3'
	D3S1209	uk	0.61	<i>Fout</i> : 5' TGGTTCACGAAGCCTAACAT 3' <i>R</i> : 5' CATGGAAGGGCCAGAGATG 3' <i>Fin</i> : 5' 6FAM-TATGTCTGCTCTTCCTCTCC 3'
	D3S1213	uk	0.61	<i>Fout</i> : 5' TGGGTAAGGTGAAGTAGC 3' <i>R</i> : 5' CGCAGGCAGTTACACAGTC 3' <i>Fin</i> : 5' PET-GTGACTGAGGAGCTATTTC 3'
	D3S1549 ^a	0.73	0.32	<i>Fout</i> : 5' TGACTCATTCAAAGCCTTGC 3' <i>R</i> : 5' CAAAGCAATGCCAATCCAC 3'
	D3S2453 ^a	0.70	0.22	<i>Fin</i> : 5' NED-GGGTTATTCACTCATTCTCC 3' <i>Fout</i> : 5' CATGTCTCTACTGCATTACTGG 3' <i>R</i> : 5' GCTACTGGCATCTAACAGGT 3' <i>Fin</i> : 5' VIC-GGGATAGAACTAACAAAGTGCT 3'

analysed with Genemapper software (Applied Biosystems, USA).

Five microlitres of exon 7 amplified inner products were purified with 2 µL of ExoSap-IT (Amersham biosciences). Minisequencing reactions were set up using the SnapShot multiplex kit (Applied Biosystems, U.S.A), following manufacturer's protocol. Resulting products were treated with Calph intestinal alkaline phosphatase (Roche) in order to remove unincorporated ddNTPs, incubating at 37°C for 1 h and heating to 75°C for 15 min to inactivate the enzyme.

Respecting the IVF cycle, the patient received a routine superovulation procedure based on the long protocol of down-regulation with gonadotrophin-releasing hormone (GnRH) agonist and ovarian stimulation with recombinant follicle stimulant hormone (FSH) for 12 days. Fourteen *cumulus-oocyte-corona* (COC) complexes were retrieved and 13 mature oocytes were obtained. Nine two-pronucleus zygotes were produced after intracytoplasmic sperm injection (ICSI). Nine cleaving embryos were available for biopsy on day 3. Two blastomeres were removed per embryo, excepting embryos number 6, 8 and 11, where only one cell was biopsied. Fourteen single blastomeres were studied by PCR. IVF, embryo culture and biopsy procedures were performed according to previously described protocols [14].

Prenatal diagnosis was carried out from amniotic liquid before reaching the 20th pregnancy week. Specific PCR amplification of exons 7 and 12 of the *PCCB* gene was performed. The PCR was carried out in a total volume of 25 µL containing 200 µM dNTP, 1.5 mM MgCl₂, 1X PCR

buffer, 1 unit *AmpliTaq Gold* polymerase (Applied Biosystems, USA) and 0.4 µM each of the forward and reverse primers. PCR conditions were as follows: a denaturation step at 95°C for 5 min. followed by 40 cycles of denaturation at 95°C for 1 min., annealing at 55°C for 1 min. and extension at 72°C for 1 min. A final extension step was performed at 72°C for 7 min. The PCR product was electrophoresed in 0.8% agarose and subsequently purified by the MultiScreen_{HTS} 961-well Filtration System (Millipore, USA). The fragments were double chain sequenced and capillary electrophoresed in an ABI 3730xl (Applied Biosystems, USA). The results were analysed and compared with the reference sequence using the SeqScape software (Applied Biosystems, USA).

Results

Four STR markers located at both sides of the *PCCB* gene were tested, resulting in two informative (D3S1209 and D3S1549). The order respect to the *PCCB* gene is: tel-D3S1549(0.32Mb)-D3S2453(0.22Mb)-*PCCB*-D3S1209 (0.61Mb)-D3S1213(0.61Mb)-cen. The analysis is shown in Fig. 1. Once the strategy of diagnosis to detect the c.737G>T and c.1218del14ins12 mutations in the *PCCB* gene was established, validation of the technique was carried out with 25 single isolated lymphocytes from each parent. Amplification failure and allelic drop out (ADO- the random non-amplification of one of the alleles) rates for

Fig. 1 Preclinical study and preimplantation genetic diagnosis (PGD) for propionic acidemia. Pedigree show data from parents and dead son involved in the informativity study and embryos from the PGD cycle. The *fine lines* represent the normal alleles for the *PCCB* gene. *Bold letter or number* represent the *PCCB* mutations present in each family member or embryos. The *dotted lines* represent the maternal c.1218del14ins12 mutation-bearing chromosome. The *discontinuous*

lines represent the paternal c.737G>T mutation-bearing chromosome. *Numbers* correspond to the STR marker alleles in base pairs. The minisequenced nucleotide position c.737 in the *PCCB* gene is indicated by G (Guanine) or T (Timidine) corresponding to normal or mutated nucleotide respectively. *ET* embryo transfer, *ADO* allelic drop out, *mat* maternal, *pat* paternal, *mut* mutation

Fig. 2 Electropherograms for some of the couple's embryos. Paternal mutation and STR linked alleles are circled. Maternal mutation and STR linked alleles are in rectangles. *G* guanine, *T* thymine

each PCR amplicon were estimated. ADO rates were 12% (3/25) for c.737G>T mutation, 0% (0/25) for c.1218del14ins12 mutation and 0% (0/50) for D3S1209 and D3S1549. PCR efficiency for each amplicon was 96% (2/50) for PCCB exon 7 and 100% for PCCB exon 12, D3S1209 and D3S1549.

PGD results are shown in Fig. 1. Total ADO and amplification efficiency rates were 17.5% (7/40) and 95% (38/40) respectively. Seven embryos were diagnosed. Six blastomeres were informative (embryos no. 2, 3 and 4), 4 cells were half informative as only one of the mutations could be diagnosed due to ADO (embryos no. 6, 8, 11 and 12) and 4 cells corresponding to embryos no. 5 and 13 remained undiagnosed as they failed to amplify, as blastomeres were lysed in the biopsy procedure and DNA did probably degrade. Electropherograms for some embryos are displayed in Fig. 2. Two embryos (embryos no. 4 and 6) reached the blastocyst stage and were transferred. A twin pregnancy was achieved.

Prenatal diagnosis revealed that the two fetuses carried the maternal mutation but none of them carried the paternal mutation, confirming the results of the PGD cycle in embryo no. 4 and clarifying the maternal mutation status of embryo no. 6.

Discussion

The aim of this case report was to establish an *ad hoc* protocol to apply PGD for propionic acidemia for the first time. PA is an autosomal recessive metabolic disease categorised in a group of disorders called organic acidemias or organic acidurias, characterised by the excretion of non-amino organic acids in urine. This results in the accumulation of highly toxic products. For this reason, the life quality of PA patients is profoundly affected. The progresses made in treatment have improved survival but have not avoided the progressive neurocognitive deterioration or long-term complications with selective organ impairment [15]. Nowadays, PGD is a well-established reproductive option for couples at risk of transmitting genetic disorders to offspring. In the context of a recessive disease like PA, at conception each sibling of a proband has a 25% chance of being affected, a 50% chance of being a carrier and a 25% chance of being unaffected and not a carrier. As carriers are asymptomatic, couples know about their genetic status when they have affected progeny.

The correlation genotype-phenotype in PA is very complex, as previously discussed [6, 16, 17]. The expression analysis of *PCCB* mutations has revealed potential correlations, although there is great phenotypic variability due to genetic heterogeneity and modulation of the effect of particular mutations by genetic and epigenetic factors.

Mutations categorised as null alleles, like ins/del mutation, are associated to severe phenotypes. On the other hand, missense mutations like G246V are associated to a mild phenotype [6]. However, many patients are compound heterozygotes, making it very difficult to predict a global phenotype. In this case report, the baby died at 2 months of age, suggesting that the confluence of these two mutations results in an early onset and severe phenotype. Nevertheless, more studies would be needed in order to establish a reliable genotype-phenotype correlation, as there are not any cases like this reported in the literature.

Respecting the PGD cycle results, ADO and amplification efficiencies rates for each STR marker obtained in the preclinical setup differ in some cases from the rates obtained in the PGD cycle. In some cases, these rates are not in line with ESHRE's guidelines (c.737G>T mutation). Nevertheless, it has been previously published that ADO and amplification efficiency rates may vary depending on the cellular type analysed [18]. In fact, total ADO rate in the informativity study was close to zero, against the 17.5% rate obtained in the PGD cycle.

In conclusion, PGD is a valid reproductive option for couples affected by rare diseases like propionic acidemia. In this case report, a novel *ad hoc* PGD protocol was applied to a couple carrying the mutations ins/del and G246V, resulting in the birth of two healthy boys.

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