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PGD for germline mosaicism

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Abstract The aim of this study was to develop and perform a preimplantation genetic diagnosis (PGD) assay discriminating between wild-type and mutant alleles in two families with germline mosaicism. Family 1 had two children affected with severe myoclonic epilepsy (*SCNA1A* del exons 1–22). Family 2 had two children with tuberous sclerosis (*TSC2* C1327T) and two healthy children. Neither mutation was detected in genomic DNA derived from the parents in either family. Informative microsatellite markers flanking *SCNA1A* and *TSC2* along with the identified mutations were used to construct haplotypes. For tuberous sclerosis, single spermatozoa were analysed using a multiplex assay that included six informative markers and the *TSC2* mutation. In family 1, deletion in the maternal allele was detected in the affected child. In family 2, both affected children and one healthy child shared the same paternal allele. To confirm mutant paternal transmission, single spermatozoa were analysed for the mutation along with six markers. Of 44 single spermatozoa, four showed the mutant T allele, allowing linkage between the mutation and the genetic markers. Both families delivered healthy children following IVF/PGD. In conclusion, germline mosaicism complicates allele assignment when constructing haplotypes for PGD. Sperm analysis is a useful tool for verifying allelic linkage.

KEYWORDS: germline mosaicism, haplotype construction, preimplantation genetic diagnosis, single-cell PCR

Introduction

Preimplantation genetic diagnosis (PGD) has been performed since the early 1990s for monogenic and chromosomal disorders (Handyside et al., 1990; Verlinsky et al., 2004b). Later on, other indications such as human leukocyte antigen matching, PGD for late-onset diseases and cancer predispositions were added (Verlinsky et al., 2004b). PGD for monogenic disorders is based on analysis of multiple microsatellite markers flanking the gene of interest in conjunction with the familial mutation (Renbaum et al., 2007). This combined analysis greatly increases the accuracy of diagnosis. Most couples presenting for PGD have familial monogenetic disorders, but some bear new mutations (Verlinsky et al., 2004a). Molecular analysis in both

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cases in based on linkage between the mutation and polymorphic informative markers flanking the mutation and requires DNA analysis from several family members, or direct sperm and polar body analysis for new mutations (Rechitsky et al., 2011). In these cases, the analysis is performed on haploid cells either before the PGD cycle for paternal de-novo mutations (Altarescu et al., 2006) or during the PGD cycle on polar bodies for de-novo maternal mutations (Altarescu et al., 2009).

This study presents two healthy couples with two affected children each. The first couple had one affected child with severe myoclonic epilepsy (Dravet syndrome) and another child who died at 2 years of age. It was presumed that both children suffered from the same genetic disorder, although DNA was available only from the living daughter. Dravet syndrome (MIM 607208), is an early onset epileptic encephalopathy characterized by generalized tonic, clonic and tonic–clonic seizures that are initially induced by fever and begin during the first year of life (Guerrini and Falchi, 2011). Seizures are usually refractory to treatment (Ceulemans, 2011). Psychomotor development stagnates around the second year of life, and affected individuals show subsequent mental decline. Since mutation in *SCN1A* can also cause the less severe disorder autosomal dominant generalized epilepsy with febrile seizures-plus, Dravet syndrome may be considered to be the most severe phenotype within the spectrum of *SCN1A*-related epilepsies (De Jonghe, 2011). In the affected daughter, a deletion, *SCNA1A* del exons 1–22, was diagnosed.

The second couple had two affected children with tuberous sclerosis and two healthy children. Tuberous sclerosis complex (MIM 191100) is an autosomal dominant multisystem disorder characterized by hamartomas in multiple organ systems, including the brain, skin, heart, kidneys and lung. Central nervous system manifestations include epilepsy, learning difficulties, behavioural problems and autism. Renal lesions, usually angiomyolipomas, can cause clinical problems secondary to haemorrhage or by compression and replacement of healthy renal tissue, which can cause renal failure (Tomasoni and Mondino, 2011). There is a wide clinical spectrum and some patients may have minimal symptoms with no neurological disability. Approximately 10–30% of cases of tuberous sclerosis are due to mutations in the *TSC1* gene, and the frequency of cases due to mutations in *TSC2* is close to 70% of all cases with a detectable mutation (Habib, 2010). *TSC2* mutations are associated with more severe disease (van Eeghen et al., 2012). Both children suffered from epilepsy due to brain hamatromas, and DNA analysis revealed a mutation C1327T in *TSC2*. The children in both families were diagnosed with mutations in autosomal dominant genes, while the parents and two healthy siblings in family 2 did not bear these mutations in their genomic DNA extracted from peripheral blood. The birth of more than one affected child with the same syndrome in a family in which both parents are healthy and do not bear the genetic mutation found in the children is indicative of germline mosaicism. Germline mosaicism (gonadal mosaicism) is a condition in which the precursor germ cells (ova or spermatozoa) are a mixture of two or more genetically different lines (Zlotogora, 1998). Germline mosaicism, has been observed in a variety of diseases including achondroplasia, Apert syndrome, Rett syndrome, tuberous sclerosis and Duchenne muscular dystrophy (Grimm et al., 1990).

Prior to a PGD cycle, a haplotype is built based on an analysis of affected and non-affected family members. Since no mutation was found in genomic DNA extracted from peripheral blood and buccal cells in either couple, haplotypes could be assigned based on genomic DNA from the parents and children but not definitively linked to the mutations. This study presents strategies to be used in PGD for cases of germline mosaicism.

Materials and methods

The first couple had two children affected with severe myoclonic epilepsy of infancy (SMEI). One of these children died at age 2 from the disease. A deletion of exons 1–22 in *SCNA1A*-was found to be the cause of the disease. This mutation was not found in the genomic DNA of the parents. The second couple had two out of four children diagnosed with tuberous sclerosis and the missense mutation C1372T in *TSC2* was identified. The mutation was also not found in the genomic DNA extracted from blood, buccal cells or a biopsy sample of a nodule considered as a periungual hamartroma of the fifth right toe of the female proband.

IVF and blastomere biopsy treatment were performed by a standard protocol involving pituitary down-regulation with gonadotrophin-releasing hormone analogue, followed by ovarian stimulation with recombinant FSH, as previously described (Altarescu et al., 2008). Blastomere biopsy was performed on day 3 at the 6–8-cell stage using the 'zona-slitting' technique (Verlinsky and Kuliev, 2005). Each blastomere was separately transferred to a 0.5 ml tube containing 5 μl of proteinase K lysis buffer (Thornhill et al., 2001).

A sample of culture medium (media blank) from each droplet that contained a biopsied blastomere was analysed to verify the absence of maternal cellular genetic material or DNA in the culture medium. In addition, a no-template control was used to monitor absence of external contamination in each PCR reaction.

Unaffected blastocysts were transferred on day 5. Surplus embryos were cryopreserved using closed-system vitrification with high-security vitrification straws (Medicult, Denmark) using equilibrium solution (7.5% ethylene glycol (EG), 7.5% dimethylsulphoxide (DMSO)) for 12–15 min and then vitrification solution (15% EG, 7.5% DMSO, 0.5 mol/l sucrose), then loaded on the straws sealed and plugged in liquid nitrogen within less than 60 s. Warming was performed using 1 mol/l sucrose, 0.5 mol/l sucrose and medium with HEPES and 20% serum for 15 min.

Molecular analysis

For each of the two genes, *SCNA1A* and *TSC2*, microsatellite markers were identified and analysed for informativity. Haplotypes based on microsatellite markers were prepared from genomic DNA using fluorescently labelled primers. For family 1, 12 markers surrounding the deletion in *SCNA1A* were found informative and used in PGD analysis (**Figure 1**): while for family 2, 11 markers surrounding *TSC2* were found informative and used in PGD analysis (**Figure 2**): Single-sperm analysis was performed using a multiplex assay that included seven