Article

Strategies for preimplantation genetic diagnosis of Angelman syndrome caused by mutations in the *UBE3A* gene

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Abstract

Angelman syndrome (AS) is a neurodevelopmental disorder associated with the loss of maternal gene expression in chromosome region 15q11-q13. AS is caused by a wide variety of genetic mechanisms, including mutations in the *UBE3A* gene that have been identified in 10–15% of patients; when the mother is heterozygous for the causative mutation, the risk of recurrence in subsequent pregnancies is 50%. The present authors have developed a preimplantation genetic diagnosis (PGD) assay for a family displaying a 10 bp deletion in exon 9 of the *UBE3A* gene, which was shared by two affected children and their phenotypically normal mother. A duplex polymerase chain reaction protocol was established, allowing the efficient amplification of the mutation together with an informative microsatellite marker (D15S122) located in intron 1 of the *UBE3A* gene. As most of *UBE3A* mutations identified so far are unique to one family, the present authors have also developed an indirect single cell protocol based upon the co-amplification of two microsatellite markers located within (D15S122) and close to the *UBE3A* gene (D15S1506). This strategy may be applied to all informative families requesting PGD for Angelman syndrome associated with mutations in the *UBE3A* gene.

Keywords: *Angelman syndrome*, UBE3A, *preimplantation genetic diagnosis, indirect analysis*

Introduction

Angelman syndrome (AS; MIM#105830) is a neurological disorder, the classical clinical features of which are severe mental retardation, delayed motor development, speech impairment, ataxia and a happy predisposition with inappropriate bursts of laughter (Williams *et al*., 1995). Other common features include epileptic seizures, microcephaly, abnormal electroencephalogram pattern, hypopigmentation and sleep disturbance. The incidence of AS is estimated to be 1 in 15 000 live births (Kyllerman, 1995), most of the cases being sporadic, although familial occurrence has often been reported (Malzac *et al*., 1998; Fang *et al*., 1999). Angelman syndrome is caused by four major molecular mechanisms involving the imprinted region 15q11-q13, which contains a number of genes expressed only from the paternally or the maternally inherited chromosome: (i) a de-novo interstitial deletion of ~4 Mb on the maternal chromosome 15 in ~70% of AS patients (Knoll, 1989); (ii) paternal uniparental disomy (UPD) for chromosome 15 in 2–5% of cases (Malcolm *et al*., 1991); (iii) imprinting defects in 3–5% of patients (Reis *et al*., 1994); and (iv) mutations in the *UBE3A* gene, also known as E6-AP, detected so far in 4–15% of AS patients (Kishino *et al*., 1997; Matsuura *et al*., 1997). Finally, another group of patients still has clinical features of AS but no detectable cytogenetic or molecular lesion of chromosome 15q11-q13.

UBE3A is a 120 kb gene composed of at least 16 exons, mapping within the 15q11-q13 region. It encodes the E6-AP ubiquitin-protein ligase involved in the ubiquitination pathway of protein degradation (Nicholls, 1998). *UBE3A* expression is mostly biallelic in a wide range of tissues (Nakao *et al*., 1994), whereas a maternal-specific expression is predominantly detected in the human brain (Vu and Hoffman, 1997; Rougeulle *et al*., 1997). More than 40 different mutations in the *UBE3A* gene have been reported to cause AS, mainly clustered in exon 9, which covers around 50% of the coding sequence, two-thirds being frameshift, leading to premature stop codons (Lossie *et al*., 2001; Kishino and Wagstaff, 1998). Mutations in *UBE3A* have been detected in 14–40% of sporadic cases and 75–80% of families with multiple affected individuals displaying a normal DNA methylation pattern (Malzac *et al*., 1998; Lossie *et al*., 2001). These mutations are found as de-novo events in half of the cases and are maternally inherited in the remainder (Fang *et al*., 1999).

The risk of recurrence depends on the genetic mechanism involved in the pathogenesis of AS and on whether the mother is a carrier of the defect, ranging from less than 1% when AS is associated to a maternal deletion or paternal UPD, to up to 50% when the identified disease-causing mechanism is a defective imprinting or a *UBE3A* mutation. Mutation analysis of *UBE3A* in familial cases is thus extremely valuable for genetic counselling and provides an opportunity for prenatal diagnosis when the mutation is also present in the mother. However, when the mutation has not been identified in the mother, there is still a risk of recurrence because of high levels of gonadal mosaicism in mothers of AS patients (Stalker *et al*., 1998), as high as 20% of sporadic and 25% of familial AS cases (Malzac *et al*., 1998); prenatal diagnosis should thus be offered to families.

Preimplantation genetic diagnosis (PGD) allows couples at risk of conceiving a genetically abnormal fetus to avoid the birth of an affected child through the selection and transfer to the mother's uterus of unaffected embryos (Handyside *et al*., 1990). The technique has been successfully applied to the study of a wide range of monogenic diseases, such as myotonic dystrophy (Dean et al., 2001), Duchenne's muscular dystrophy (Girardet *et al*., 2003a), hereditary retinoblastoma (Girardet *et al*., 2003b) and polycystic kidney disease (Verlinsky *et al*., 2004), among others. In some cases, the genetic composition of oocytes may be tested through biopsy and analysis of the first and second polar bodies (Verlinsky and Kuliev, 2000), which may be followed by biopsy of the resulting embryo without any effect on the viability of that embryo (Magli *et al*., 2004). Furthermore, the genetic analysis of individual spermatozoa through their duplication has also been recently proposed in order to test the outcome of male meiosis (Willadsen *et al*., 2003).

Presented here is the first PGD strategy for a family with AS found to carry a 10 bp deletion in exon 9 of the *UBE3A* gene. A specific duplex polymerase chain reaction (PCR) protocol was developed in order to specifically study the disease-causing mutation together with an intragenic microsatellite. Furthermore, as most of the *UBE3A* mutations identified so far are unique to one family, and because the setting-up of a mutation-specific diagnosis on single cells is time-consuming and labour-intensive, the present authors also report a single-cell PCR protocol allowing the analysis of two microsatellite markers within and close to the *UBE3A* gene, which may be applied to all informative families requesting PGD for AS associated with mutations in the *UBE3A* gene.

Materials and methods

Patients' history

A couple presented for PGD because they had a daughter and a son affected with AS (**Figure 1**). A 10 bp deletion in exon 9 of the *UBE3A* gene (2037del10) had been previously identified in the affected children (II.1 and II.2) that was also shared by their asymptomatic mother (I.2) (Malzac *et al*., 1998). A recurrence risk of 50% was therefore established. After the identification of the disease-causing mutation in this family, two pregnancies investigated by standard prenatal diagnosis protocols at the first trimester followed; a healthy boy was born and one pregnancy was selectively terminated as the fetus was a carrier of the mutation.

After extensive counselling, the couple gave informed consent for PGD and subsequent genetic tests. This study was approved by the local multidisciplinary network licensed for prenatal diagnosis.

To confirm the genetic status of the couple and to establish a single-cell PCR assay, peripheral blood samples were collected from the five members of the family. Genomic DNA was extracted using standard protocols, then haplotyping analysis was performed using primers for four microsatellite markers in the 15q11-13 region: D15S122 located within intron 1 of the *UBE3A* gene, and D15S210, D15S1506 and D15S113 flanking UBE3A. Their heterozygosity rates were around 85, 69, 79 and 73%, respectively.

At the time of the first PGD cycle, the woman was aged 37 and her husband was 36 years old.

Lymphocyte isolation and cell lysis

Single cell isolation was performed as previously described (Girardet *et al*., 2003a). Briefly, lymphocytes were separated by centrifugation through Ficoll-Pâque from 5–10 ml of fresh blood collected into heparinized tubes. Single lymphocytes were aspirated using a mouth-controlled glass microcapillary under an inverted microscope, rinsed twice in phosphate-buffered saline, then transferred to microtubes containing 3 µl alkaline lysis buffer (200 mmol/l potassium hydroxide, 50 mmol/l dithiothreitol) (Cui *et al*., 1989). For every five single cells collected, an aliquot from the last washing medium was transferred to a PCR tube to serve as a blank control. Single cells and blanks were lysed by incubation at 65°C for 10 min, then immediately used for amplification or frozen at –20°C until PCR was performed.

Polymerase chain reaction

Protocol I: 2037del10 and D15S122

To control for an eventual allele dropout (ADO) in clinical PGD procedures, it was decided to include an informative marker in addition to the disease-causing mutation in the