PRENATAL DIAGNOSIS
Prenat Diagn 2004; **24**: 302–306.
Published online in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/pd.841

Pregnancy after preimplantation genetic diagnosis for Sanjad–Sakati syndrome

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Sanjad–Sakati syndrome (SSS) is an autosomal recessive disorder characterized by congenital hypoparathyroidism, growth and mental retardation. In Saudi Arabia, the disease is caused by a deletion of 12 bp (155-166nt) in the tubulin-specific chaperone E gene. In a family with two affected siblings with SSS, preimplantation genetic diagnosis (PGD) was performed. Fluorescent PCR (F-PCR) was utilized to check the heterozygosity and the homozygosity status of the parents and the affected children, respectively. F-PCR was then optimized for single-cell analysis by using peripheral blood lymphocytes. The patient underwent a cycle with intra-cytoplasmic sperm injection. A total of 11 embryos were obtained and biopsied. There were five heterozygous, three homozygous affected and three normal embryos. One heterozygous and one normal embryo were transferred because of their very good quality (morula). A singleton pregnancy was obtained, and amniosynthesis confirmed the presence of the heterozygous fetus. These results show for the first time, the feasibility of PGD for SSS. Copyright © 2004 John Wiley & Sons, Ltd.

KEY WORDS: PGD; ICSI; PCR; Sanjad–Sakati; Kenny–Caffey

INTRODUCTION

Sanjad–Sakati syndrome (SSS) is an autosomal recessive disorder characterized by congenital hypothyroidism with growth and mental retardation accompanied with seizures (Hershkovitz *et al*., 1995). The children suffering from SSS have typical facies with deep-set eyes, depressed nasal bridge with beaked nose and micrognathia. Immune deficiency in some patients has resulted in neonatal sepsis and/or repeated infections (Sanjad *et al*., 1991; Kalam and Hafeez, 1992). The disease is quite frequent in Saudi Arabia and is distributed in certain Bedouin tribes spread all over the country. The incidence of SSS in Saudi Arabia is estimated at 1 in 40 000 to 1 in 100 000 live births.

The tubulin-specific chaperone E (*TBCE*) gene encodes one of several chaperone proteins required for the proper folding of α-tubulin subunits and the formation of α-β-tubulin heterodimers, which is a very important component of cilia, flagella, the mitotic spindle and other cellular structures. The *TBCE* gene was found carrying 12-bp deletion in patients suffering from SSS (Parvari *et al*., 2002). The gene locus is mapped on the chromosome 1 long arm (1q42-43) (Parvari *et al*., 1998). The deletion 155-166del is the only one found causing the disease in Saudi Arabia.

All SSS patients described to date have been of Middle Eastern origin. However, the clinical findings of SSS are reminiscent of those seen in Kenny–Caffey syndrome (KCS), a similar syndrome with the additional features of osteosclerosis and recurrent bacterial infections (Diaz *et al*., 1999).

Preimplantation genetic diagnosis (PGD) is an alternative for prenatal diagnosis for couples who have a high risk of transmitting inherited disease to their offspring. PGD is performed on one or two single blastomeres biopsied from 4 to 10 cell embryos on day 3 after fertilization (Handyside *et al*., 1990). The possibility of selecting and transferring only the unaffected embryos to the uterus is an alternative to elective abortion, following prenatal diagnosis of an affected fetus.

To our knowledge, this report describes for the first time the PGD on a family affected by the reported deletion of SSS.

PATIENTS AND METHODS

Patients

A Saudi family with two affected SSS boys was referred for PGD. Parents and siblings were studied to check the presence of the only known mutation. Peripheral blood was drawn from each family member to extract genomic DNA and isolate single lymphocytes for mutation identification and single-cell PCR optimization.

Methods

PCR procedure for mutation detection

Genomic DNA was extracted using Dnazol kit (Invitrogene, USA) and analyzed for the presence of the single 155–166 deletion, the only known mutation (Parvari *et al*., 2002). The following PCR protocol was used: 100 ng of DNA, 2 µL of 5′ 6-FAM labeled primers (10 µM), dNTP (2.5 mM), $MgCl_2$ (50 mM), PCR buffer 10X and one unit of Taq polymerase (Invitrogene, USA). PCR products were analyzed on ABI 310 genetic analyzer (Applied Biosystems, USA).

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Received: 16 August 2003
Revised: 8 October 2003
Accepted: 11 December 2003

Single-cell optimization on lymphocytes

Twenty single leukocytes from parents and 10 single leukocytes from the affected children were loaded in PCR tubes containing 5 μL of lysing buffer (1% detergent, 1X PCR buffer and 20 mg/mL of proteinase K). Aliquots from the last washing droplets were taken to serve as blanks (2 tubes). Samples and blanks were incubated at 45 °C for 15 min, followed by proteinase inactivation at 96 °C for 20 min. Cell lysates were used directly for amplification or stored at −80 °C.

ICSI procedure

Controlled ovarian hyperstimulation, oocyte retrieval and intra-cytoplasmic sperm injection (ICSI) procedure were performed as previously described (Coskun *et al*., 2000).

Embryo biopsy

The zona pellucida was pierced by a stream of acidified Tyrode's (Medicult, Denmark) using a fine needle (Hardy *et al*., 1990). One blastomere was gently aspirated through the hole with the help of an embryo biopsy pipette. After biopsy, the blastomeres were checked for the presence of a nucleus and transferred to a 0.5-mL PCR tube containing 5 μL of the lysing buffer. PCR tubes were incubated at 45 °C for 15 min and at 96 °C for 20 min.

PCR procedure for PGD

PCR reaction mix was added to the cells to a final volume of 20 μL and final concentration, 50 mM KCl, 100 mM tris-HCl pH 8.3, 2 mM $MgCl_2$, 0.1-mg/mL gelatin, 0.2 mM dNTP, 1 μM primers (6FAM-5′-ATCCCGAGAGAGGAAAGCAT-3′ forward; 5′-TCTT CCTCTCCATCCCTCCT-3′ reverse) and 1.25 U Taq polymerase (Invitrogene, USA). PCR was carried out on a Biometra thermocycler using the following program: 5-min denaturation at 96 °C, followed by 40 cycles of 20 s at 96 °C, 60 s at 55 °C and 20 s at 72 °C, followed by 6 min at 72 °C. The PCR products were analyzed on an ABI 310 genetic analyzer.

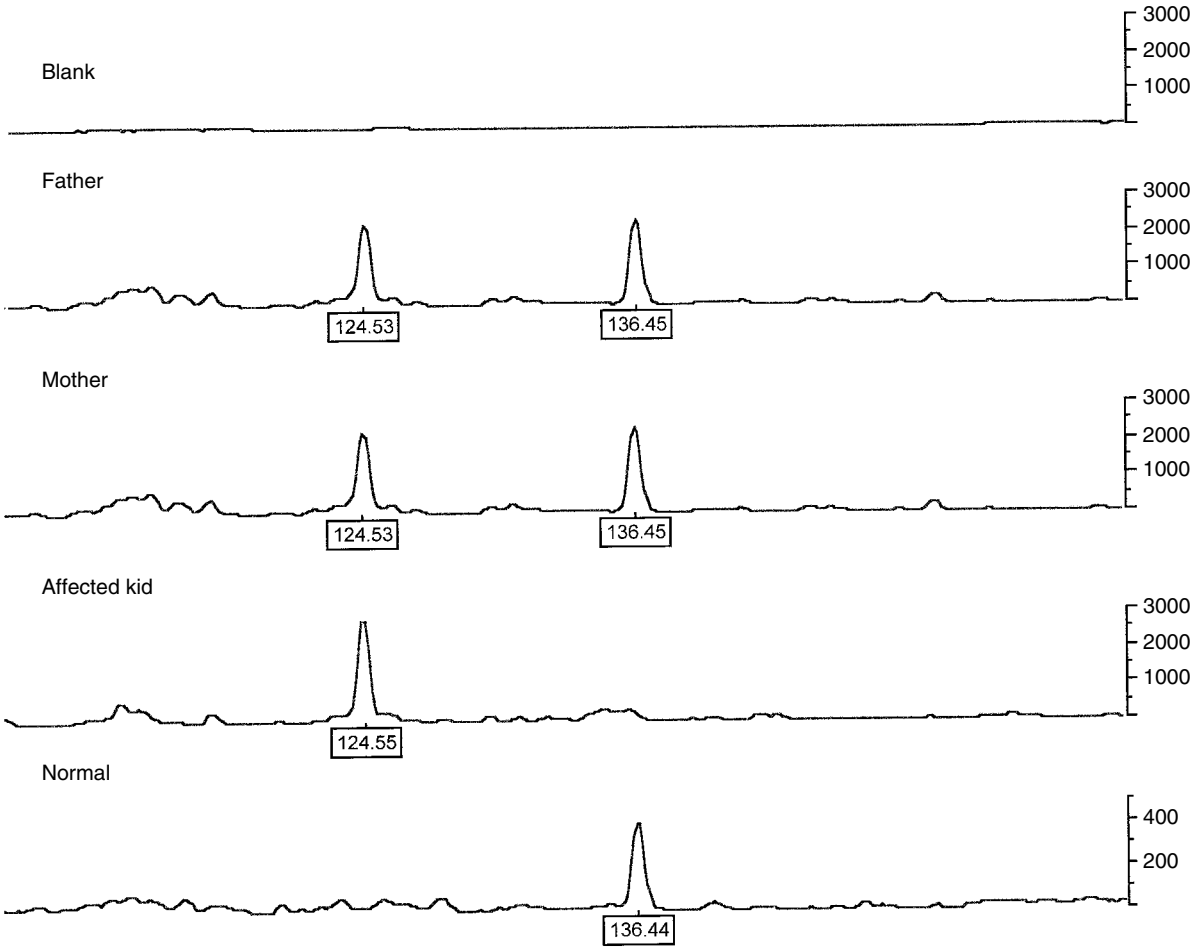

Figure 1—Fluorescent PCR on lymphocyte DNA of the SSS family. The heterozygosity in respect to the 12-bp deletion could be seen in father and mother (2 peaks), while the affected and the normal child are homozygous affected and normal respectively. The size in bp is mentioned below the peak

Prenatal diagnosis procedure

Prenatal diagnosis was performed on genomic DNA and 10 single amniotic cells that were placed in 10 different tubes containing lysing buffer; the single-cell PCR procedure described above was performed.

RESULTS

Genomic DNA diagnosis showed that parents are carriers of the 12-bp deletion (155–166nt) of *TBCE* gene, while the two affected children were homozygous for this deletion (Figure 1).

Single lymphocytes derived from the parents (20) and children (10) were diagnosed in respect to the deletion found in the peripheral blood. The result showed complete matching with 0% ADO rate in the lymphocyte amplified. Three lymphocytes (two from parent and one from the affected child) showed absence of amplification (Table 1).

The female partner produced 20 ovarian follicles (leading follicular diameter of 19 mm), 16 oocytes were retrieved, and all were suitable for ICSI. A total of 11 were fertilized, and all embryos were biopsied successfully on day 3 of culture, where 8 embryos were at the 8-cell stage, 1 at the 7-cell stage and 2 at the 4-cell stage.

The same protocol for single lymphocytes was applied to blastomeres. F-PCR results showed five heterozygous, three normal, one homozygous affected blastomeres and two failure of amplification, which were confirmed as homozygous affected along with the abnormal one (Figure 2). On day 4, following oocyte retrieval, two embryos at the morulae stage (one heterozygous and one normal) were chosen for transfer and one singleton pregnancy was obtained.

Amniosynthesis was performed to check the PGD diagnosis. Ten single cells were isolated from the amniotic sample and tested for the mutation of 155–166del, in addition to genomic DNA from amniotic cells. The result showed seven heterozygous cells and three failure of amplification (Figure 3).

DISCUSSION

SSS is a disease incompatible with normal life. Since the mutation is known, the use of PGD as a preventive tool instead of prenatal diagnosis would be an ideal option for the affected families.

Table 1—Summary of PCR amplification results on single lymphocytes and amniotic cells. ADO and FA correspond to allelic drop out and failure of amplification respectively

	Total	Diagnosed	ADO	FA (%)
Single lymphocytes	30	27	0	3 (10%)
Amniotic cells	10	7	0	3 (30%)
Total number of cells	40	34	0	6 (15%)

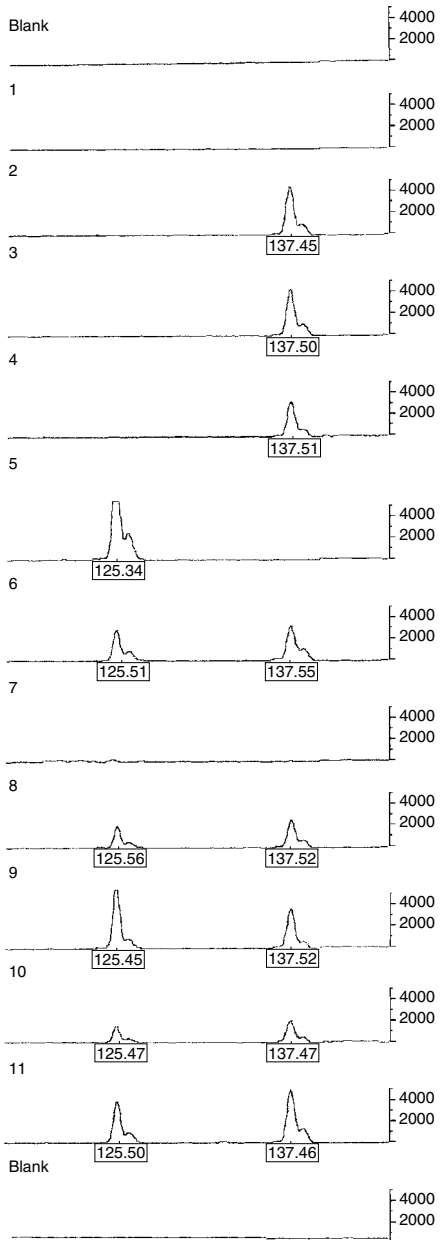

Figure 2—Result from the clinical preimplantation genetic diagnosis (PGD) for SSS. Two blank samples are used as amplification negative controls, lanes 1 to 11 are the blastomeres collected from the 11 biopsied embryos. Failure of amplification could be seen in blastomeres 1 and 7. Normal blastomeres correspond to lanes 2, 3 and 4. Abnormal and heterozygous blastomere are represented by lane 5 and 6, 8, 9, 10 and 11 respectively

Conventional nested PCR is a powerful tool to amplify DNA from single cells (Hellani *et al*., 2002). However, this method is prone to carry over contamination, a major factor for misdiagnosis. The use of F-PCR in one amplification round could minimize its occurrence and avoid additional PCR steps (Moutou *et al*., 2001). This technique was used in our PGD cycle successfully with regard to single-cell DNA amplification.

ADO is a phenomenon encountered in single-cell PCR. For an autosomal recessive disorder, ADO does not lead to serious clinical misdiagnosis. This will

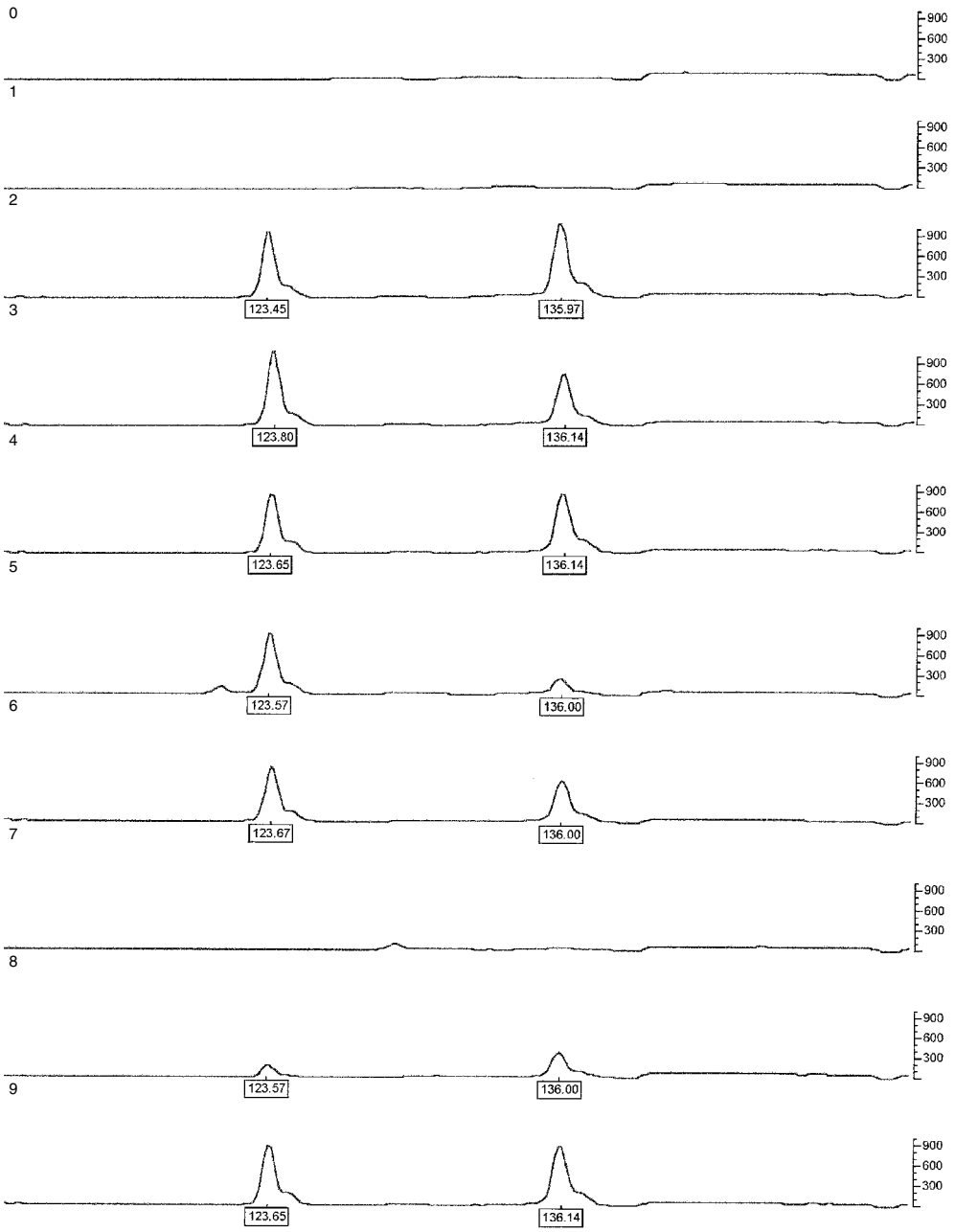

Figure 3—Use of single-cell PCR on amniotic cells in order to confirm the PGD diagnosis. Lanes 0, 1, 7 show failure of amplification, the rest of the cells show heterozygosity of the fetus in respect to SSS mutation

result in transferring of a heterozygous embryo as normal or discarding of a heterozygous embryo as abnormal. In a situation where the number of embryos generated is low, heterozygous embryos become important for transfer and should not be discarded. Along with the current PGD cycle, we performed a single-cell PCR on 20 parental heterozygous lymphocytes and 10 amniotic cells. Since PCR on an amniotic cell DNA showed heterozygous result for SSS mutation, amniotic single cell could be added to the total number of heterozygous cells amplified by PCR, which becomes 30. In these 30 heterozygous cells, ADO was absent.

Since ADO and contamination are the most important limitations of single-cell PCR that might lead to misdiagnosis, the use of linked markers, proven to lower their frequency, should be adopted. In cases where there is no linked marker available (consanguineous population), unlinked highly polymorphic loci might be used to detect contamination (Harper *et al*., 2002). This strategy seems to be efficient, and not using it could be the major limitation of our protocol, since, even with one round F-PCR, contamination from maternal cumulus cells or an external source cannot be detected. Therefore, we suggest that contamination markers should be incorporated in order to make the protocol less error prone.

Preferential amplification (PA) is another parameter that could be seen in single-cell PCR (Findlay *et al*., 1995). PA could be observed when there is a great difference in the amplification of two alleles. In the case of conventional nested PCR, PA might have been considered as ADO. The use of F-PCR could prevent such assumption. In our case, PA was observed only during prenatal diagnosis and was not present in lymphocytes or blastomeres. This might suggest that PA occurrence is cell related.

Failure of amplification (FA) is another parameter that could interfere with PGD results. In order to estimate the FA rate, the 30 heterozygous cells tested should be added to the 10 homozygous affected lymphocytes, which were amplified for the affected children. The number of amplification failures obtained were five in heterozygous (two lymphocytes and three fetal cells) and one in homozygous affected lymphocytes with an overall rate of 15%. FA may not only be due to PCR failure but also due to the cell quality or the loading problem (Thornhill *et al*., 2001).

Failure of amplification in blastomere PCR (18%) was similar to that found in lymphocytes and amniotic cells (15%). ADO in blastomeres is more problematic. In the case of heterozygosity, there is no concern about ADO since both alleles are amplified. However, the apparent homozygosity (normal or abnormal) could be due to ADO. Homozygous embryos, which were discarded, were easily identified. However, normal ones were either transferred or frozen, and could not be confirmed until they become fetuses. We assume that ADO does not exist in our blastomere PCR because (i) the percentage of distribution (3/11 are normal, 3/11 are abnormal and 5/11 are heterozygous) is in accordance with the autosomal recessive disease inheritance, and (ii) the ADO in the 30 single heterozygous cell tested is absent. Therefore, the occurrence of ADO is unlikely.

On day 4, two embryos (one normal and one heterozygous) reached the morula stage. After consultation with patients and their pediatricians, these two morulas were chosen for transfer. SSS is a serious inherited disease, but heterozygous individuals have no clinical manifestations (Gul *et al*., 2000). Indeed, neither parent had SSS or any other clinical manifestations.

The prenatal diagnosis test performed on amniotic cells showed heterozygosity of the fetus. This fetus could be derived from the heterozygous embryo transferred or the normal embryo, which could be heterozygous due to ADO. Since all the indications suggest an absence of ADO, as discussed above, the transferred heterozygous embryo probably implanted and became the fetus. It is of interest to notice the importance of having a low ADO rate, since such a heterozygous embryo would have been missed if, due to ADO, it was diagnosed as affected.

The similarity between SSS and the autosomal recessive form of KCS extends beyond the clinical features. Indeed, the same deletion in the *TBCE* gene is responsible for Kenny–Caffey disease (Parvari *et al*., 2002). Therefore, PGD protocol for SSS could be easily applied to KCS.

This work, as far as we know, is the first to report PGD for SSS. F-PCR has been utilized successfully. This technique could be applicable to families suffering from KCS since they share similar mutation and clinical manifestations. PGD is ethically more acceptable than standard prenatal diagnosis, as it obviates the need for abortion, which might not be acceptable or available. The high rate of consanguinity amongst the Arab population and specifically that in Saudi Arabia leads to a high incidence of serious recessive disorders. Such diseases require expensive treatment or are untreatable. SSS treatment is estimated at $15 000 per patient per year in our hospital. PGD is considered to be the best option to avoid transmission of this and many other genetic diseases.

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