Fluorescent PCR and automated fragment analysis in preimplantation genetic diagnosis for 21-hydroxylase deficiency in congenital adrenal hyperplasia

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Congenital adrenal hyperplasia (CAH) is an autosomal recessive disease which is most often caused by a deficiency in steroid 21-hydroxylase. The disease is characterized by a range of impaired adrenal cortisol and aldosterone synthesis combined with an increased androgen synthesis. These metabolic abnormalities lead to an inability to conserve sodium and virilization of females. The most common mutation causing the severe form of CAH is a conversion of an A or C at nucleotide (nt) 656 to a G in the second intron of the steroid 21-hydroxylase gene (CYP21) causing aberrant splicing of mRNA. A couple was referred to our centre for preimplantation genetic diagnosis (PGD) for 21-hydroxylase deficiency in CAH. A PGD was set up to detect the nt656 A/C → G mutation using fluorescent polymerase chain reaction (PCR) and subsequent restriction enzyme digestion and fragment analysis on an automated sequencer. Using DNA or single cells from the father, the normal allele could not be amplified. Non-amplification of the normal allele has been previously described in asymptomatic carriers, therefore the PCR was further developed using heterozygous lymphoblasts from the mother. The PCR was shown to be highly efficient (96% amplification), accurate (0% contamination) and reliable (0% allelic drop-out). The couple started PGD treatment and the second PGD cycle resulted in a twin pregnancy. The genotype of the fetuses was determined in our laboratory using chorionic villus sampling material using the method described here. Both fetuses were shown to be heterozygous carriers of the mutation, and two healthy girls were born.

Key words: 21-hydroxylase deficiency/congenital adrenal hyperplasia (CAH)/fluorescent PCR analysis/pre-implantation genetic diagnosis (PGD)

Introduction

Congenital adrenal hyperplasia (CAH) is a common (1:10 000 to 1/18 000 live births) human genetic disease which in 90% of the cases are caused by a deficiency in the enzyme steroid 21-hydroxylase (Donohoue et al., 1995). This autosomal recessive disease is characterized by a wide range of impaired adrenal cortisol and aldosterone synthesis combined with an increased androgen synthesis. The defect in the cortisol and aldosterone synthesis leads to an inability to conserve sodium (‘salt wasting’), which can be fatal if left untreated. The adrenal overproduction of androgen precursors leads to prenatal masculinization (ambiguous genitalia) in females and postnatal virilization in both sexes. Three major disease phenotypes can be distinguished: (i) the classic salt-wasting form; (ii) the classic simple virilizing form; and (iii) the non-classic form. In the classic salt-wasting form, cortisol and aldosterone production is severely impaired and is accompanied by an overproduction of androgen precursors. In the classic simple virilizing form, cortisol synthesis is impaired but aldosterone synthesis is normal to elevated (non-salt wasting). These patients also undergo virilization. In the non-classic form, there is a subtle defect in cortisol synthesis and aldosterone production is normal. The androgen precursors are moderately elevated in these patients, but this is clinically important only in pubertal or adult females.

The treatment of patients with the simple virilizing form includes glucocorticoid therapy and surgical correction of the ambiguous genitalia of the females. Patients having the salt-wasting form of the disease require an additional treatment with mineralcorticoids. Adult male patients with CAH appear to be fertile, on the basis of their sperm counts and reproductive histories (Urban et al., 1978). A high number of adult female patients lack sexual activity (Federman, 1987). Those who have had surgical correction and have an adequate introitus show a low fertility rate: 7% in the salt-wasting form and 60% in the virilizing form (Mulaikal et al., 1987).

The gene encoding 21-hydroxylase (CYP21) is located on 6p21.3. The molecular genetic basis of the disease has been thoroughly investigated (White, 1989; Strachan et al., 1990). The mutations can be divided into different groups according to severity, which makes it possible to predict the clinical outcome in affected subjects on the basis of genotyping (Speiser et al., 1992; Wedell, 1998). The majority of the mutations causing the deficiency are the result of recombination processes between CYP21 and a highly homologous closely linked pseudogene CYP21P. In fact, most of these mutations...
are gene conversions between CYP21P and CYP21, so that the mutation(s) that normally inactivate(s) the pseudogene are instead found in the normally expressed or active gene (Higashi et al., 1986; White et al., 1986). The most common conversion is a mutation in the second intron at nucleotide (nt)656, in which an A or C in CYP21 is replaced by a G from CYP21P (Higashi et al., 1991), resulting in aberrant splicing of mRNA. The nt656 A/C→G mutation is associated with both the severe salt-wasting form and the simple virilizing non-salt-wasting form of the disease (Speiser et al., 1992). A previously described problem when amplifying the nt656 region is the absence (non-amplification) of the normal allele in healthy carriers resulting in an affected genotype (Day et al., 1996).

Polymerase chain reaction (PCR)-based genotyping has facilitated genetic counselling of families with CAH. A couple with a child diagnosed for 21-hydroxylase deficiency was referred to our centre for preimplantation genetic diagnosis (PGD). Both parents carry the mutation at nt656. Here we describe the development of a single cell PCR for the detection of the nt656 splice site mutation in the CYP21 gene causing 21-hydroxylase deficiency in CAH. The second PGD cycle led to a twin pregnancy of two carrier babies which was confirmed at prenatal diagnosis using the method developed for PGD. Two healthy girls were born.

Materials and methods

Patient description

A young non-consanguinous couple (woman 34 years old, man 39 years old) was referred to our centre for PGD because of a risk of 21-hydroxylase deficiency (25%). She was Gravitas8 Partus 2 Abortus 6 . Their first child, a boy, had been born and was shown to be affected by the disease. Seven pregnancies followed, three miscarried spontaneously, three were terminated after prenatal diagnosis and one healthy boy was born. Both parents carry the most common mutation of CYP21 in the second intron at nt656. In addition, the father and the affected boy carry the Val281Leu mutation in exon 7 of the same allele.

Collection of single lymphoblasts

Peripheral blood lymphocytes from both partners and the affected child were transformed with Epstein Bar Virus. The lymphoblasts were cultured according to standard procedures (Ventura et al., 1988). Single lymphoblasts were collected as described (Sermon et al., 1986). Briefly, a few colonies were aspirated and transferred to a 1.5 ml Eppendorf tube. The cells were washed three times with phosphate-buffered saline (PBS). Finally, they were resuspended in 50 l PBS and kept at 4°C. Single lymphoblasts were washed three times in 3 l microdrops of Ca2+- and Mg2+-free medium (136.8 mM NaCl, 2.7 mM KCl, 0.4 mM Na2HPO4, 5.6 mM glucose, 3.5 mM EDTA, 11.9 mM NaHCO3, 0.01% w/v Phenol Red) supplemented with 15 mg/ml bovine serum albumin (BSA) in a Petri dish using fine hand-drawn micropipettes (30 l; Drummond Scientific Company, USA). They were transferred blindly by mouth controlled pipetting to 2.5 l alkaline lysis buffer (ALB) (200 mM KOH, 50 mM dithiothreitol) in 200 l PCR tubes. An aliquot from the last washing droplet was transferred to a PCR tube containing ALB to serve as a blank per five cells collected. The samples were stored (not longer than 1 week) at –80°C until further processing.

Stimulation and oocyte preparation

Ovarian stimulation was carried out by a desensitizing protocol using the gonadotrophin-releasing hormone (GnRH) agonist, buserelin (Suprefact, Hoechst, Brussels, Belgium) combined with human menopausal gonadotrophin (HMG, Humegon; Organon, Oss, The Netherlands) and human chorionic gonadotrophin (HCG; Pregnyl, Organon; or Profasi; Serono, Brussels, Belgium). Intravaginally administered progesterone (Utrogestan; Piete, Brussels, Belgium) was used for luteal phase supplementation. The details of this stimulation protocol have already been reported (Ubaldi et al., 1995).

Oocytes were retrieved by vaginal ultrasound-guided puncture of ovarian follicles 36 h after HCG administration. Oocyte demudation was performed as described using enzymatic and mechanical procedures (Van de Velde et al., 1997).

Semen preparation

The partner’s semen was collected in a sterile container after masturbartion. Sperm concentration and motility were evaluated according to the recommendations of the World Health Organization (WHO, 1992). Sperm morphology was determined after Shorr staining (WHO, 1992) of a smear using the strict Tygerberg criteria (Kruger et al., 1986). The freshly ejaculated semen was prepared by washing only (De Vos et al., 1997).

ICSI procedure

Intracytoplasmic sperm injection (ICSI) was used for fertilization to prevent contamination with sperm cells (Lissens et al., 1997). The preparation of the holding and injection pipettes has been described in detail elsewhere (Van Steirteghem et al., 1995; Joris et al., 1997). Only metaphase II oocytes were injected. The ICSI procedure has been described in detail elsewhere (Van Steirteghem et al., 1995). Oocytes were held by the holding pipette with the polar body at the 6 o’clock position and the injection pipette was inserted into the oocyte at the 3 o’clock position (Nagy et al., 1995). After injection, the oocytes were washed and stored in 25 µl microdrops of B2 medium under oil in a Petri dish. They were kept in an incubator at 37°C in an atmosphere of 5% CO2, 5% O2 and 90% N2.

Assessment of survival, fertilization and embryo development

The morning after injection (16–22 h later), the oocytes were checked for survival and fertilization (Nagy et al., 1994). The numbers and aspects of polar bodies and pronuclei were recorded. The criteria for normal fertilization were the presence of two individualized or fragmented polar bodies together with two clearly visible pronuclei. Embryo cleavage and quality were evaluated 2 and 3 days after ICSI as described: type A (0% anucleate fragmentation), type B (1–20% fragmentation), type C (21–50% fragmentation) and type D (>50% fragmentation) (Van de Velde et al., 1997). On day 3, only transferable embryos (0–50% anucleate fragmentation) were considered for biopsy.

Embryo biopsy

Biopsy was performed on day 3 in the morning as described (De Vos et al., 1998). The embryo was fixed by the holding pipette and a hole was made in the zona pellucida by a stream of acidic Tyrode’s (pH 2.3) using a fine needle (5–7 l inner diameter). Most of the embryos were already compacted and were, therefore, preincubated in Ca2+- and Mg2+-free medium for 5 min. If the embryo had six or fewer cells, only one blastomere was aspirated. If the embryo had seven or more cells, two blastomeres were removed. The blastomeres were gently aspirated through the hole using a pipette with an inner diameter of 40–45 µm and were released next to the embryo. They were checked for the presence of a nucleus. Single blastomeres were
were not purified and digested, they were simply applied onto the gel.

HCl, 10 mM MgCl$_2$, 1 mM dithiothrietol and 100

Pharmacia Biotech, Gent, Belgium. The results were processed using

phoresis unit used was an ALF Automated DNA Sequencer from

sequencing gel; Immunosource, Zoersel, Belgium). The electro-

acrylamide (19:1), 7 M urea, Tris Borate Electrophoresis buffer,

of 50 mM KCl, 10 mM Tris–HCl pH 8.3, 2 mM MgCl$_2$, 0.1 mg/ml

the lysed cells to a final volume of 25

(Eurogentec, Seraing, Belgium). The reaction mix was added to

lysed cells to a final volume of 25 µl and final concentrations

of 50 mM KCl, 10 mM Tris–HCl pH 8.3, 2 mM MgCl$_2$, 0.1 mg/ml gelatin, 0.2 mM dNTP (Pharmacia, Gent, Belgium), 0.4 µM primers

and 1.25 IU Taq polymerase (Perkin Elmer, Brussels, Belgium). The

PCR programme was as follows: 5 min at 96°C; 10 cycles of 96°C

for 30 s, 61°C for 30 s and 72°C for 30 s; followed by 37 cycles of 94°C for 30 s, 61°C for 30 s and 72°C for 30 s; followed by 7 min at 72°C.

**Purification and digestion**

The PCR product was purified using the High Pure PCR Product

Purification Kit (Boehringer Mannheim, Germany) using the manufactur-

er’s protocol, with some small modifications. Briefly, 125 µl

binding buffer was added to the 25 µl PCR product and mixed

thoroughly. The mixture was centrifuged for 30 s at 14 000 g on a

High Pure filter tube combined with a collection tube. The flow-

through was discarded and the filter was washed twice with 500 and

200 µl respectively of wash buffer by centrifugation for 30 s on

14 000 g. Finally, the filter plate was placed in a new Eppendorf tube

and the DNA was eluted with 50 µl sterile distilled H$_2$O by centrifugation for 30 s at 14 000 g.

The sample was digested using the restriction enzyme MwoI (New England Biolabs Inc), which cuts into a 5'-GCN6GC...3' site. Purified

PCR product (10 µl) was incubated for 1 h at 60°C in a final volume of

20 µl restriction buffer containing 150 mM NaCl, 50 mM Tris–

HCl, 10 mM MgCl$_2$, 1 mM dithiothrietol and 100 µg/ml BSA. Blanks

were not purified and digested, they were simply applied onto the gel.

**Electrophoresis**

Restricted PCR product (6 µl) was mixed with 6 µl loading buffer

(10 mM Tris–HCl pH 7.6 and 1 mM EDTA and 5 mg/ml Dextran

Blue 2000 in formamide) and loaded on the gel (6% acrylamide/bis-

acrylamide (19:1), 7 M urea, Tris Borate Electrophoresis buffer,

Sequencing gel; Immunosource, Zoersel, Belgium). The electrophoresis unit used was an ALF Automated DNA Sequencer from

Pharmacia Biotech, Gent, Belgium. The results were processed using

the Fragment Analyser software provided by the manufacturer.

**Results**

In a first step, the genotype of the family members was

confirmed. The PCR was set up using a primer set consisting of a fluorescently labelled forward primer and a reverse primer

overlapping the 8 bp deletion region in the pseudogene CYP21P

avoiding amplification of the pseudogene. The total PCR

product is 185 bp. The mutation at nt656 is recognized by

restriction enzyme digestion using MwoI which cuts the

mutated allele into two fragments: 65 bp (which is not

fluorescently labelled and which would coincide with the

primer peak because it is too small) and 120 bp.

DNA (100 pg) was amplified and digested. The normal

control DNA showed one 185 bp peak after digestion indicating

that the pseudogene CYP21P, which contains the mutation, is

not amplified by this primer set (Figure 1a, lane 5). Both

parents were shown to have the 120 bp affected peak and the

normal 185 bp peak and were thus confirmed as carriers of

the mutation (Figure 1a, lanes 3 and 4). Unexpectedly, their

affected child was also shown to be heterozygous for the mutation

(Figure 1a, lane 2). Based on the idea that some PCR

reagents might interfere with the digestion reaction, the DNA

was purified before digestion using a DNA purification kit as

described above. The normal control DNA showed one 185

bp peak after purification and digestion (Figure 1b, lane 5), whereas

the affected child now appeared to have only the affected 120 bp peak (Figure 1b, lane 2). The mother was

confirmed to be heterozygous (Figure 1b, lane 4), however the

healthy father appeared to be homozygous for the affected gene although he has no clinical signs of the disease (Figure

1b, lane 3). Although non-amplification of the normal allele

at nt 656 is a well known phenomenon, we have tried to

investigate this technical problem by using more DNA (1 µg),

AmpliTaq Gold polymerase instead of AmpliTaq polymerase

and a different primer set. We have not been able to obtain

the heterozygous genotype of the father.

Using single cell PCR on lymphoblasts, similar results were

obtained (Table I). The overall amplification rate was 96%

(72/75 lymphoblasts) and contamination was not observed

(0/25 blanks). To determine the allelic drop-out (ADO), 30

single lymphoblasts from the mother were analysed. Of these

cells were amplified, 29 (97%) and none of them showed

ADO (0%) (Table II). Single cells (n = 15) from the father

were also analysed, 15 cells were amplified (100%) and

they all showed the phenomenon of non-amplification of the

normal allele.

Despite the above technical problem with regard to the

father’s genotype, the couple started PGD treatment because

the preferential amplification of the affected allele would lead

only to misdiagnosis of a healthy heterozygous embryo, which

would appear affected and would not be transferred. In the

first PGD cycle (Table II) 10 cumulus–oocyte complexes

(COCs) were retrieved which were mature and those were

all injected. The semen characteristics were normal (76% progressive motility and 18% normal morphology). The next

morning, seven oocytes showed normal fertilization. On the

third day, seven blastomeres were retrieved from six embryos

(two embryos had reached the 8 cell-stage, two embryos had

reached the 6-cell stage and two embryos were only at the

4-cell stage). Six out of seven cells were amplified (86%). Two

embryos were homozygous normal, two were heterozygous

and one was homozygous affected. In one embryo no result was

obtained, but it later turned out to be heterozygous. The
diagnosis of the affected embryo was confirmed later. No ADO was found in this cycle. Two homozygous normal embryos and one heterozygous embryo were transferred, and the remaining heterozygous embryo was frozen. At the moment of transfer, only the heterozygous embryo had cleaved further. The patient did not become pregnant in this cycle.

In the second cycle (Table II), 12 COCs were obtained. Mature oocytes (n = 10) were injected (normal semen parameters with 67% progressive motility and 22% normal morphology), and six were normally fertilized the next morning. On day 3, six embryos were available for biopsy (all but one had reached the 7–8-cell stage). All the cells (11 blastomeres) were amplified (100%) so that diagnosis was obtained for all embryos: one normal, three heterozygous and two affected. In this cycle there was no ADO. In one embryo, two blastomeres from the embryo were heterozygous while one of the two blanks was contaminated. The genotype of this contaminating peak could not be tested later. This embryo was not transferred because the presence of a contaminating normal allele might have led to the transfer of an affected embryo. The embryo was frozen. The diagnosis of the affected embryos was confirmed later. The homozygous normal embryo was transferred together with two heterozygous embryos. None of these embryos had cleaved further between the time of biopsy and the moment of transfer. This PGD cycle resulted in a twin pregnancy. The diagnosis was confirmed in our laboratory after chorionic villus sampling and genotype analysis using the method described here to detect the mutation. The

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**Figure 1.** Results of the polymerase chain reaction (PCR)-based genotyping of DNA from the family after fluorescent PCR, (a) without and (b) with DNA purification, before restriction enzyme digestion and fragment analysis on an automated sequencer: lane 2, the patient; lane 3, the father; lane 4 the mother; lane 5, an unrelated normal control. Lane 6 represents undigested PCR product from the patient, and lane 1 represents a molecular weight standard showing 100, 150, 200 and 250 bp peaks. The x axis shows the running time in minutes.

**Table I.** Results of the polymerase chain reaction (PCR)-based genotyping of lymphoblasts from the family and an unrelated normal control after fluorescent PCR

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Genotype</th>
<th>Amplification (%)</th>
<th>Contamination (%)</th>
<th>ADO (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient</td>
<td>homozygous affected</td>
<td>14/15 (93)</td>
<td>0/5 (0)</td>
<td>NA</td>
</tr>
<tr>
<td>Father</td>
<td>heterozygous</td>
<td>15/15 (100)</td>
<td>0/5 (0)</td>
<td>NA</td>
</tr>
<tr>
<td>Mother</td>
<td>heterozygous</td>
<td>29/30 (97)</td>
<td>0/10 (0)</td>
<td>0/29 (0)</td>
</tr>
<tr>
<td>Control</td>
<td>homozygous normal</td>
<td>14/15 (93)</td>
<td>0/5 (0)</td>
<td>NA</td>
</tr>
</tbody>
</table>

ADO = allelic drop-out; NA = not applicable.
Implanted embryos were healthy carriers of the nt656 mutation and were dizygous. Two healthy girls were born after 36 weeks of pregnancy.

Discussion
Steroid 21-hydroxylase deficiency causing congenital adrenal hyperplasia is an autosomal recessive disease that is characterized by salt wasting and virilization of female fetuses. The molecular basis of the disease is diverse, the most common mutation is a conversion of an A or C at nt656 to a G in the second intron of the gene encoding 21-hydroxylase causing impaired mRNA splicing.

We describe here an efficient (amplification was 96% using lymphoblasts and 96% during PGD) and accurate (contamination was 0% using lymphoblasts and 6% during PGD) PCR for the PGD of the nt656G/G mutation in the CYP21 gene by using fluorescent PCR, DNA purification, restriction enzyme digestion and fragment analysis on an automated sequencer.

In our experience, it is unusual to insert a DNA purification step between the PCR and the restriction enzyme digestion. The MwoI enzyme is probably inhibited by reagents from the PCR mix.

An additional problem was the finding that the husband was genotyped as homozygous affected for the nt656 mutation although he showed no clinical signs of the disease. The problem was also not solved by using a different primer set, excluding the possibility that the non-amplification had been due to polymorphism of one of the PCR priming sites. The normal allele was also not amplified using a hot-start PCR system with AmpliTaq Gold polymerase. Most probably, the normal allele is not amplified (i.e. is ‘dropped-out’) during the reaction, leading to an ‘asymptomatic’ affected result from an individual carrying a normal allele that remains undetectable because of preferential amplification of the allele carrying the nt656 splicing mutation. This phenomenon has already been described (Day et al., 1996), and it was found that in pedigrees of 150 patients with 21-hydroxylase deficiency caused by the nt656 splicing mutation, 13 first-degree family members were genotyped as affected but showed no clinical signs of CAH. ADO is a major problem of single-cell PCR (Findlay et al., 1995), but we found that the normal allele also failed to be amplified after PCR using 1 µg of DNA. The non-amplification of the normal allele could be investigated further, by use of microsatellite haplotyping (Day et al., 1996), but this has not been done because it would not be of any use for the PGD. Absence of the normal allele would never lead to transfer of an affected embryo but would result in the misdiagnosis of a healthy heterozygous embryo that would appear affected and consequently would not be transferred. In addition, the undetectable normal allele is not necessarily absent in the offspring of the ‘asymptomatic’ affected parent because it can appear again in the next generation (Day et al., 1996).

Consequently, the ‘ADO’ obtained using lymphoblasts from the father was 100%. We assume that non-amplification of his normal allele is due to a technical problem specific for his DNA and, therefore, this number does not represent the drop-out caused by the PCR technique. Therefore, we prefer to use the term ‘non-amplification’ when discussing results obtained using cells or DNA from the father.

The real ADO was determined using heterozygous lymphoblasts from the mother and was shown to be 0%. This finding makes the test reliable because the probability of transferring an affected embryo after misdiagnosis by ADO on two blastomeres is low.

For these reasons, the PGD treatment was started. In the first cycle, two homozygous normal embryos and one heterozygous embryo were transferred, but the patient did not become pregnant. In the second cycle, one homozygous normal embryo and two embryos carrying the nt656 mutation were transferred. In all, nine embryos were analysed: three were homozygous normal, six were heterozygous and three were affected (expected ratio 25/50/25). There was no ADO in these PGD cycles, but the group of the affected embryos might contain one or more heterozygous embryo(s) with an undetectable normal allele that was not amplified. The second cycle resulted in a twin pregnancy of two carrier fetuses.

In conclusion, we developed a PGD for the detection of 21-hydroxylase deficiency in CAH caused by the nt656A/C(G) splicing mutation in the CYP21 gene using fluorescent PCR, DNA purification, restriction enzyme digestion and fragment analysis on an automated sequencer. Non-amplification of the normal allele can occur, resulting in the misdiagnosis and subsequent loss of healthy heterozygous embryos that appear affected for the nt656 mutation.

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References


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