Pregnancy after preimplantation genetic diagnosis for Ataxia Telangiectasia

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Ataxia Telangiectasia (AT) is an autosomal recessive disorder with an incidence estimated at 1 in 40 000 to 1 in 100 000 live births. More than 100 different somatic and germ-line mutations have been identified in the AT gene, the majority of which cause premature protein truncation. The immense size of the AT gene (66 exons) complicates the detection of mutations. A Saudi family with three affected children suffering from AT consulted our IVF centre for preimplantation genetic diagnosis (PGD). Despite advanced maternal age and unknown mutation, the family was screened for AT mutations. A large deletion in the gene was found to be responsible for the phenotype of AT. The mutation detection permitted us to perform PGD on AT for the first time. Single cell PCR consisted of amplifying one of the deleted exons, exon 19. Homozygous affected embryos show an absence of the exon, while in heterozygous or normal embryos the exon is amplified successfully. After ICSI, three embryos were suitable for embryo biopsy. After biopsy only one embryo showed exon amplification and was transferred. A singleton pregnancy ensued and prenatal diagnosis confirmed the presence of exon 19. This report demonstrates that PGD is feasible despite advanced maternal age and poor response to follicle stimulation.

Key words: Ataxia Telangiectasia/embryo/ICSI/PCR/PGD

Introduction

The Ataxia Telangiectasia (AT) gene responsible for ataxia telangiectasia (Savitsky et al., 1995, 1997) is an autosomal recessive disorder characterized by cerebellar ataxia and progressive neutromotor degeneration, immune deficiency, and the appearance of dilated blood vessels in the eyes and face (telangiectasias). Patients also manifest growth retardation, premature ageing of skin and hair, chromosomal instability, lymphoreticular malignancies and acute sensitivity to ionizing radiation. The protein-coding region of the AT gene contains 9168 bp in 66 coding exons spread over 146 kb of genomic DNA (Platzer et al., 1997). The AT gene displays a complex mutational spectrum. Thus far, more than 100 different somatic and germ-line mutations have been identified, the majority of which cause premature protein truncation (Gilad et al., 1996). The large size and genomic structure of the AT gene greatly complicates the process of screening genomic DNA samples for all possible sequence variations.

Preimplantation genetic diagnosis (PGD) is an alternative to 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 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 PGD to detect AT. A Saudi family with three AT affected children was accepted in our PGD programme. Since the mutation causing the AT phenotype was unknown, we screened the family to determine the mutation involved. After mutation identification, we performed a single cycle of PGD and an ongoing pregnancy was obtained. Prenatal diagnosis has confirmed the PGD diagnosis.

Patients and methods

Patients

Three AT patients from the affected family were studied; Table I summarizes their clinical features. The carrier parents were first cousins. Peripheral blood was drawn from the family to extract DNA and isolate single lymphocytes for mutation identification and PGD test optimization.

PCR procedure for mutation detection

In order to assess the mutation causing the AT phenotype, genomic DNA was analysed at the following exons: 5, 6, 8, 10, 14, 16, 17, 19, 25, 27, 33, 63 and 64. The primers and PCR conditions used were as described previously (Vorechovsky, 1996). Briefly, AT exons were amplified from 100 ng of DNA using specific primers (10 μmol/l), dNTP (2.5 mmol/l), MgCl2 (50 mmol/l), PCR buffer 10× and one unit of Taq (5 IU/μl) polymerase (Life Technology, USA). PCR products were analysed on 2% agarose gels containing ethidium bromide and visualized under UV light.

PGD test optimization on lymphocytes

A total of 10 single leukocytes from each of both parents and the affected children was collected in a 0.5 ml PCR tube containing 5 μl of lysing buffer (1% Tween 20, 1% triton 100X, 1X PCR buffer and 20 mg/ml proteinase K) (Verlinsky and Kuliev, 2000). Aliquots from the last washing droplets were taken to serve as blanks (five 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.
but that the other three exons (27, 33, 64) were absent. This result showed that exons 6, 8, 10, 14, 16, 27, 33 and 64. Our results showed that the five first exons (6, 8, 10, 14 and 16) were present, but that the other three exons (27, 33, 64) were absent. This result led us to focus on exons 17, 18, 19, 20 and 21. After amplification of these exons, it was found that exons 17 and 18 were present while exons 19 and above were absent in the affected children (Figure 1).

Single lymphocytes were randomized and coded to perform a blinded nested PCR analysis. All cells gave the correct genotype, i.e.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Father</th>
<th>Mother</th>
<th>A3</th>
<th>A4</th>
<th>A5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malignancy</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Radio sensitivity</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>4+</td>
<td>4+</td>
<td>4+</td>
</tr>
<tr>
<td>Age (years)</td>
<td>50</td>
<td>42</td>
<td>23</td>
<td>17</td>
<td>12</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>Onset (years)</td>
<td>–</td>
<td>–</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Gait ataxia</td>
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<td>–</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td>Limb ataxia</td>
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<td>–</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Dysarthria</td>
<td>–</td>
<td>–</td>
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<td>Yes</td>
<td>Yes</td>
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<tr>
<td>Chorea and/or dystonia</td>
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<td>–</td>
<td>Yes</td>
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<td>Yes</td>
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<tr>
<td>Eye movements</td>
<td>–</td>
<td>–</td>
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<td>Yes</td>
<td>Yes</td>
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<tr>
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<td>–</td>
<td>–</td>
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<td>No</td>
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<tr>
<td>Infection</td>
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<td>–</td>
<td>Mild</td>
<td>Mild</td>
<td>Mild</td>
</tr>
</tbody>
</table>

### ICSI procedure

Ovarian stimulation, oocyte retrieval and decoration, and the ICSI procedure were performed as previously described (Coskun et al., 2000).

### Embryo biopsy

A hole was made in the zona pellucida by a stream of acidic Tyrode’s (Medicult, Denmark) solution and introduced into the embryo. 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 PCR lysing buffer and the nested PCR procedure described above was performed. PCR tubes were incubated at 45 °C for 15 min at 96 °C for 20 min.

### PCR procedure

The reaction mixes for PCR were decontaminated by UV exposition for 15 min. Reaction mix was added to the cells to a final volume of 20 µl and final concentration of 10% dimethylsulphoxide (DMSO), 50 mmol/l KCl, 100 mmol/l Tris–HCl pH 8.3, 2 mmol/l MgCl₂, 0.1 mg/ml gelatin, 0.2 mmol/l dNTP, 1 µmol/l primers (forward: GTTGTGCCCTTCTCTTATGTT; reverse: ACTCTACTATTGATCGAGA) and 1.25 IU Taq polymerase (Life Technology). PCR was carried out on a Biometra thermocycler using the following programme: 5 min denaturation at 96 °C followed by 28 cycles of 20 s at 96 °C, 60 s at 55 °C and 20 s at 72 °C, and completed by 6 min at 72 °C. An aliquot of 2 µl from the first PCR was seeded into a second round PCR reaction. The reaction mix had the same final concentrations and volume as the first round PCR mix except that the inner primers (forward: CTTGAAACATCTTTGTTTCTCTCTCTCT; reverse: GTAAATACATATTCTTACTCAGA) were used. The PCR programme for the second round was 5 min denaturation at 96 °C followed by 30 cycles of 20 s at 96 °C, 60 s at 55 °C and 20 s at 72 °C, completed by 6 min at 72 °C. The PCR products were separated on a 2% agarose gel, run at 100 V for 1 h.

### Prenatal diagnosis procedure

Prenatal diagnosis was performed on DNA extracted from chorionic villus tissue. Briefly, DNA was extracted by a DNAzol kit (Life Technology). A total of 100 ng of DNA was used for PCR with the exon 19 primers for the second round of the nested PCR. Moreover, five single cells separated from chorionic villus sampling (CVS) were placed into five different tubes containing lysing buffer and the nested PCR procedure described above was performed.

### Results

Genomic DNA from one of the affected children was used to screen for the presence of exons 6, 8, 10, 14, 16, 27, 33 and 64. Our results showed that the five first exons (6, 8, 10, 14 and 16) were present, but that the other three exons (27, 33, 64) were absent. This result led us to focus on exons 17, 18, 19, 20 and 21. After amplification of these exons, it was found that exons 17 and 18 were present while exons 19 and above were absent in the affected children (Figure 1).

Although many exons were found to be deleted in this family, we chose to analyse a single exon, exon 19, by PCR amplification in our conventional nested PCR. The results from the analysis of the three embryos, as well as the positive and negative controls, are shown in Figure 2. Only the blastomere isolated from embryo 3 produced an amplification band for exon 19 and thus only embryo 3 was suitable for embryo transfer. The results were obtained on day 3 and the transfer was performed on day 4. The embryo was at the morula stage when it was transferred, which is consistent with it being a good quality embryo as it was at the 8-cell stage on day 3.

Conventional prenatal diagnosis at 12 weeks gestation was used to confirm the PGD diagnosis. DNA extracted from CVS material was tested for the presence or the absence of exon 19. In addition, five single cells were isolated from the CVS culture and tested by nested PCR using external and internal primers for exon 19. The presence of the exon 19 band in the DNA sample and in four of the five cells tested confirms that the fetus did not inherit the AT defect segregating in the family. However, it remains to be determined whether this fetus is a carrier or is homozygous unaffected with respect to the AT gene (Figure 3).

### Discussion

We report here the identification of a large 3’ deletion in a family with AT. This identification allowed us to offer PGD to this couple with three affected children to avoid a pregnancy with AT. The deletion is likely to be the cause of AT in this family as it causes complete absence of the AT protein (data not shown). The deletion starts after exon 18 and continues until the presumed end of the AT gene, but stops short of the marker D11S1778. As there is no other report of genes between the end of the AT gene and this marker we presume that the deletion does not affect other genes. Most of the existing reports describing AT mutations mention mutations or small deletions which lead to truncated proteins (Gilad et al., 1996; Wright et al., 1996). We believe this is the first report showing the deletion of more than two-thirds of the AT gene.

Gatti et al. have reported that carriers of AT could be classified into two categories, those with a predisposition to cancer and those without, depending on the nature of the deletion in the AT gene (Gatti et al., 1999). Those with a predisposition to cancer have altered protein functions, while those without a predisposition have deletions which result in the absence of the protein. In the family under study, the mutated AT gene caused the absence of the majority of the AT protein. In addition to this there was no clinical evidence for a predisposition to cancer in the family (Table 1).

Allelic drop-out (ADO) is defined as the amplification failure of one of the two alleles in a heterozygous cell. Since the mutation involved in this report is a deletion, ADO, if it occurred, would be observed in the lymphocytes corresponding to carrier parents as a failure of amplification. None of the 20 parent lymphocytes tested showed failure of amplification, indicating that the percentage of ADO is very low. Moreover, the embryos diagnosed as affected with respect to exon 19 of the AT gene were re-tested and shown to inherit...
Figure 1. PCR amplification products of exons 17, 18, 19, 20 and 21 of the AT gene. Father and mother are both carriers; A3 and A4 are two of their affected children. The absence of exons 19, 20 and 21 is shown.

Figure 2. Nested PCR amplification exon 19 (300 bp) from single blastomeres isolated from each of three embryos. Positive signals from embryo 3 and a blastomere from an embryo not carrying the deletion (positive control) are shown. The other two blastomeres (1 and 2) did not produce a band for exon 19 and thus were unsuitable for transfer. No bands were seen in Blank, B. A 100 bp PCR marker was used (Sigma).

Figure 3. Nested and normal PCR of exon 19 (300 bp) from CVS single cells and DNA from CVS tissue confirm the PGD diagnosis. The results for five fetal cells (FC1–5), fetal DNA (F DNA) and positive (+) control, marker and negative control (Blank, B) are shown.

Contamination is the other major obstacle in PGD leading to serious misdiagnosis. In this diagnostic situation, a false positive result caused by contamination would lead to the transfer of an affected embryo. The reliability of our PGD analysis regarding contamination was based on the absence of a band in all blank tubes tested and in each of homozygous affected cells, lymphocytes and blastomeres.

Our PGD analysis was unable to differentiate between carrier or homozygous normal embryos. However, the absence of a predisposition to cancer allowed us to transfer either heterozygous or normal
embryos. Finding a linked genetically informative marker would have allowed us to avoid transferring carrier embryos. We tested four markers, all of which were found to be non-informative. Moreover, due to the age of the female patient (42 years) and the expectation that the number of embryos suitable for biopsy would be low, the use of a linked marker was deemed less important. Indeed, the couple received a transfer of only a single embryo and conventional prenatal diagnosis confirmed the presence of a healthy fetus. The deletion observed by PCR for the remaining two embryos was confirmed by analysing the remainder of the blastomeres from these embryos (data not shown).

The decision to cancel a PGD cycle is a serious responsibility. The decision is usually made by the clinical team as the couple will decide to continue the cycle in most cases. The case presented here would have been cancelled if we had followed the strict criteria for cancellation used by some centres (Vandervorst et al., 1998, Santalo et al., 2000) as the age of the mother was 42 years and only five oocytes were suitable for injection. However, we elected to continue the cycle based on two facts: (i) the good quality of the embryos obtained, giving the couple a good chance of pregnancy (Heller et al., 1998); and (ii) the reproductive history of the family which had suffered for 22 years with three affected children.

This work, as far as we know, is the first to report PGD for AT and may give hope to couples who find themselves in a similar situation. This approach is ethically acceptable while standard prenatal diagnosis, that necessitates therapeutic abortion, is not. The high rate of consanguinity among the Arab population in general and specifically in Saudi Arabia leads to a high incidence of deleterious recessive disorders. As many diseases are either untreatable or necessitate expensive treatments, an alternative for preventing affected children is required. PGD is considered to be the best option so far to prevent the large genetic burden facing many couples.

References

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