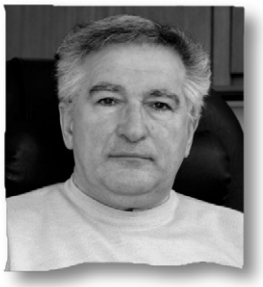
Symposium: Genetic and epigenetic aspects of assisted reproduction

Preimplantation genetic diagnosis: technological advances to improve accuracy and range of applications



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Abstract

Preimplantation genetic diagnosis (PGD) is an option for couples who are at risk that enables them to have unaffected progeny without facing the risk of pregnancy termination after prenatal diagnosis as currently practiced. It is also one of the practical tools used in assisted reproduction technology to improve the chance of conception for infertility cases with poor prognosis. Because PGD is performed using a single biopsied cell, technological advances are important to improving PGD accuracy. This has contributed to the avoidance of misdiagnosis in PGD for single gene disorders, and extensive experience in PGD for chromosomal disorders suggests strategies for more reliable evaluation of the chromosomal status of the preimplantation embryo. This paper describes the present status of PGD for genetic and chromosomal disorders, its accuracy and range, and how PGD is an integral part of IVF and genetic practices.

Keywords: *accuracy, chromosomal disorders, preimplantation genetic diagnosis, preimplantation HLA testing, single gene disorders*

Introduction

Preimplantation genetic diagnosis (PGD) is currently an established procedure, allowing couples who are at risk of transmitting genetic disease to have an unaffected offspring without facing prenatal diagnosis and termination of pregnancy.

The detection and avoidance of transfer of embryos with genetic abnormalities is also an alternative to the traditional selection of embryos based on morphological criteria. Because no correlation exists between chromosomal status and embryo morphology, the latter may no longer be an acceptable selective criterion, as morphologically normal embryos may be found to be chromosomally abnormal in IVF practice, destined to be lost during implantation and post-implantation development (Gianaroli *et al*., 2001; Verlinsky *et al*., 2004a; Munné *et al*., 2007).

PGD is based on oocyte or embryo biopsy and DNA analysis of the biopsied material by polymerase chain reaction (PCR) or fluorescent in-situ hybridization (FISH). The biopsy procedures presently range from sampling of the first and second polar body (PB1 and PB2, respectively) to single blastomere removal at the cleavage stage or to blastocyst biopsy, which also provides the possibility of a confirmatory diagnosis following PB or blastomere analysis (De Boer *et al*., 2004; Verlinsky and Kuliev, 2005). Although the methods involved in achieving PGD accuracy will differ depending on circumstances, in many occasions a reliable diagnosis can be achieved by using two or three different methods, especially when more than one indication for PGD is involved, such as PGD for single gene disorders with human leukocyte antigen (HLA) typing, or preimplantation HLA typing together with aneuploidy testing. Even for some single indications, such as chromosomal aneuploidy, the reliability of PGD cannot be sufficient if performed solely by either PB1 and PB2 biopsy or embryo biopsy. Paternally derived chromosomal abnormalities, such as sex chromosome aneuploidies, will be missed by PB analysis, while mosaicism and/or uniparental disomies deriving from trisomies originating from female meiotic errors will be missed in embryo biopsy. With the expanding range of PGD indications, combined testing is required when testing is performed for causative gene, linked markers, HLA typing and aneuploidy in the same case. Therefore, single, double or even triple biopsy may be required in order to establish an accurate PGD, provided that the additional biopsy procedures have no detrimental effect (Cieslak *et al*., 2006).

PGD for inherited disorders

PGD is presently applied to 170 different conditions, with the most frequent indications still being cystic fibrosis, haemoglobin disorders and dynamic mutations. However, the range of indications is gradually expanding to include the risk for common diseases with genetic predisposition, as well as non-genetic conditions, such as HLA typing with the purpose of stem cell therapy for the affected siblings in the family (Verlinsky and Kuliev, 2006). The available experience has greatly improved the accuracy of single cell DNA analysis for monogenic and chromosomal disorders.

Almost half of PGD cases for single gene disorders were performed for X-linked disorders, originally carried out by gender determination with either PCR or FISH technique. This is, however, no longer acceptable, as it involves a potential discard of 50% of normal male embryos. On the other hand, DNA sequence information is becoming available for an increasing number of these disorders, allowing a specific diagnosis and transfer of mutation-free male or heterozygous female embryos. Performing a specific diagnosis also avoids embryo biopsy, completing the diagnosis by PB analysis only. As X-linked disorders are maternally derived, the embryos derived from mutation-free oocytes require no further testing and may be transferred irrespective of gender or the paternal genetic contribution (Verlinsky *et al*., 2002a).

The PB approach also provides the prospect of pre-embryonic diagnosis, which is required in many population groups where objection to the embryo biopsy procedures makes PGD non-applicable. The first pre-embryonic genetic diagnosis has recently been realized for Sandhoff disease in a couple with a religious objection to embryo destruction (Kuliev *et al*., 2006). Although pre-embryonic genetic diagnosis could have previously been done by PB1 testing only (Verlinsky *et al*., 1992; Munné *et al*., 1998; Montag *et al*., 2004; Magli *et al*., 2006), it is not actually sufficient for the accurate prediction of embryo genotype without PB2 analysis. However, PB2 analysis should be done prior to pronuclear fusion (syngamy) to ensure that only zygotes originating from mutation-free oocytes may be allowed to progress to embryo development. Such embryos may be transferred either in the same or a subsequent menstrual cycle, avoiding the formation and possible discard of any affected embryo.

Pre-embryonic diagnosis has become a reality with the possibility of completing the genetic analysis in a time frame prior to pronuclear fusion. In the first original case mentioned, 18 oocytes were obtained in a standard IVF protocol, from which PB1 were removed as usual 4–5 h after aspiration, followed by PB2 sampling, approximately 6 h after ICSI. The analyses for maternal mutation in the biopsied PB1 and PB2 were completed in less than 9 h, while all the oocytes were still at the pronuclear stage, and the affected oocytes were frozen at this particular stage, prior to embryo formation. The embryos originating from the mutation-free oocytes were allowed to develop and were replaced after reaching the blastocyst stage. This resulted in a singleton pregnancy and birth of a healthy mutation-free child, demonstrating the feasibility of PGD application in those ethnic groups in which no embryo testing and discarding is presently acceptable (Kuliev *et al*., 2006).

Significant improvement in the accuracy of PGD has been achieved with developments in the detection of preferential amplification and allele-specific amplification failure (allele drop out: ADO), the main potential causes of PGD misdiagnoses. Clearly, the failure of detection of one of the mutant alleles in double heterozygous blastomeres due to ADO will lead to misdiagnosis in compound heterozygous embryos. This no longer presents a problem with the application of protocols for the simultaneous detection of the causative gene, together with highly polymorphic markers, closely linked to the gene tested (Verlinsky and Kuliev, 2005). With a sufficient number of linked markers amplified together with the gene tested, a risk for misdiagnosis may be substantially reduced or even practically eliminated. The protocol involves a multiplex nested-PCR analysis, with the initial first-round PCR reaction containing all the pairs of outside primers, followed by amplification of separate aliquots of the resulting PCR product with the inside primers specific for each site. Following the nested amplification, PCR products are analysed either by restriction digesting, real-time PCR, direct fragment size analysis, or minisequencing. Depending on the mutation studied,

different primer systems are designed, with a special emphasis on the elimination of false priming to possible pseudogenes, for which purpose the first-round primers are designed to anneal to the regions of non-identity with the pseudogene.

Misdiagnosis due to preferential amplification and ADO in single cell molecular analysis may also be avoided by using a direct fragment size analysis of PCR product by fluorescence (F-PCR) and real-time PCR, which may reduce the ADO rate almost by half compared with conventional PCR analysis. F-PCR also allows a simultaneous gender determination, DNA fingerprinting and detection of common aneuploidies. Because F-PCR is much more sensitive than standard gel detection, fewer PCR cycles are run, with multiplex reactions performed and analysed in one tube.

The need for post-PCR processing is also eliminated in real-time PCR, which detects specific nucleic acid amplification products as they accumulate by using a fluorescently labelled oligonucleotide probe. Real-time PCR allows screening for genes with a single base-pair variation between normal and mutant genotypes, and detecting sequence changes, deletion or insertion. One of the advantages of real-time PCR is also that the PCR tubes are not opened after the amplification reaction is complete, since all the data have already been collected. This prevents contamination by PCR products and reduces the number of false positive results. The method can be further improved by performing real-time PCR with molecular beacons instead of using linear probes in TaqMan systems.

Earlier attempts to perform whole genome amplification prior to DNA analysis appeared to be impractical because of the possibility of ADO during this initial procedure. However, the recent introduction of multiple displacement amplification seems to be more accurate and also makes it possible to preserve a PCR product for further testing (Handyside *et al.*, 2004; Hellani *et al.*, 2005).

To further improve the reliability of the test, especially in PGD for dominant conditions or pre-selection of normal or heterozygous embryos in testing for autosomal recessive disorders, the current strategy is not only to exclude the presence of the mutant gene but also to confirm the presence of the normal allele(s) by haplotype analysis. Although a sufficient number of informative closely linked markers are usually available for performing multiplex PCR, this still might not be the case in performing PGD by conventional PCR analysis in some ethnic groups. One of the approaches for avoiding misdiagnosis in such cases may be sequential PB1 and PB2 testing, which is the option for testing for maternally derived mutations in any case. Detection of both mutant and normal alleles in the heterozygous PB1, along with the mutant allele in the corresponding PB2, leaves no doubt that the resulting maternal contribution to the embryo is normal, even without testing for the linked markers as a control. However, the mutation-free status of the oocytes may not be predicted reliably if the corresponding PB1 is homozygous, unless the absence of one of the alleles in this PB1 could be excluded by simultaneous testing for linked polymorphic markers (Verlinsky and Kuliev, 2005).

It has also been shown that the reliability of PGD is affected by a high rate of aneuploidy in oocytes and embryos and by an extremely high prevalence of mosaicism at the cleavage stage (Gianaroli *et al.*, 2001; Munné, 2002; Munné *et al.*, 2002; Kuliev *et al.*, 2003). The fact is that the lack of a mutant allele tested may simply be determined by the lack of one of the homologous chromosomes on which the causative gene is located. Simultaneous testing for the causative gene together with a specific chromosome number, by adding primers for chromosome-specific microsatellite markers to the multiplex PCR reaction, may be useful. All of these requirements make it necessary to develop a specific PGD design for each couple before performing the actual PGD; this may also require single sperm typing to establish the paternal haplotypes, so that the linked marker analysis may be performed in addition to mutation testing, especially in cases of paternally derived dominant conditions (Verlinsky and Kuliev, 2006).

Presently available protocols allow accurate PGD for complex cases that require testing for two or three different mutations, especially when the affected maternal partner has two different mutations. As mentioned, paternal haplotyping using single sperm may be required in such cases in order to reliably exclude the risk of misdiagnosis due to potentially undetected ADO of a paternal mutant allele; this is demonstrated in the PGD performed for the couple recorded here who were at risk of producing a child with cystic fibrosis. The couple had had two previous pregnancies, the first one resulting in spontaneous abortion of twins and the second terminated following prenatal diagnosis, which identified an affected fetus. PGD was performed for three different mutations in the *CFTR* gene, namely R117H and G542X from the mother, and 1717 from the father. In addition, multiplex PCR analysis performed for the above three mutations was combined with testing for age-related aneuploidy because of the mother's advanced reproductive age. Multiplex hemi-nested PCR was performed on blastomeres from 14 embryos, allowing simultaneous detection of the paternal and maternal CFTR haplotypes, as well as chromosomal status of the embryos. Six embryos were predicted to be carriers based on the presence of the maternal mutation and the normal paternal allele. Two that were also free of aneuploidy were replaced, resulting in pregnancy and birth of a healthy child who was predicted and confirmed to be the carrier of maternal mutation G542X (Verlinsky and Kuliev, 2006).

PGD generally requires knowledge of sequence information for Mendelian diseases, although it may also be performed when the exact mutation is unknown. It may be expected that, with the expanded use of single nucleotide polymorphisms (SNP), linkage analysis will allow PGD for any genetic disease irrespective of the availability of specific sequence information (Verlinsky *et al.*, 2004b). This approach might be more universal, making it possible to track the inheritance of the mutation without actually testing for the gene itself.

Ongoing developments may soon allow the genetic analysis of single biopsied cells by DNA microarray technology, which may also provide the possibility of simultaneous testing for a causative gene, multiple linked markers, health-related genetic variability and chromosomal abnormalities in the same biopsied PB or blastomere. The feasibility of applying microarray technology at the single cell level is presently also being assessed, with a clear prospect of PGD for single gene disorders using microarrays, especially with simultaneous detection of mutations and surrounding SNP.

Expanding range of PGD indications

The above developments made PGD applicable to a wider range of disorders, including dynamic mutations, genetic predisposition for late-onset disorders and preimplantation HLA matching. PGD for late-onset disorders with genetic predisposition was first applied for a couple with an inherited cancer predisposition, determined by *p53* tumour suppressor gene mutations (Verlinsky *et al*., 2001a). Traditionally, these conditions have not been considered as an indication for prenatal diagnosis that would lead to pregnancy termination, which is not justified on the basis of genetic predisposition. Instead, the possibility of choosing embryos for transfer that are free of genetic predisposition would obviate the need for considering pregnancy termination because only potentially normal pregnancies are established. Although the application of PGD for these conditions is still controversial, it is presently being performed for an increasing number of disorders with genetic predisposition that present beyond early childhood and may not even occur in all cases, such as inherited forms of cancers (Rechitsky *et al*., 2002), Alzheimer disease (Verlinsky *et al*., 2002b) and congenital malformations (Abou-Sleiman *et al*., 2002; Verlinsky *et al*., 2003, 2005a; He *et al*., 2003).

One of the attractive current indications is preimplantation HLA typing. The first case of preimplantation HLA typing was performed in combination with PGD for Fanconi anaemia complementation group C, which resulted in a successful hematopoietic reconstitution in the affected sibling by transplantation of stem cells obtained from the HLA-matched offspring resulting from PGD (Verlinsky *et al*., 2001b). To improve the access to the HLA-identical bone marrow transplantation in sporadic bone marrow failures, this approach was then applied with the sole purpose of ensuring the birth of the HLA-identical offspring not involving PGD, which also resulted in radical treatment of a sibling with a sporadic Blackfan-Diamond anaemia by stem cell transplantation from an HLA-identical child born following preimplantation HLA typing (Verlinsky *et al*., 2004c). Eventually, preimplantation HLA typing has become one of the most attractive indications for PGD, performed currently with or without testing for the causative gene (Kahraman *et al*., 2004a; Rechitsky *et al*., 2004; Van de Velde *et al*., 2004; Kuliev *et al*., 2005).

However, because most patients requesting preimplantation HLA typing are of advanced reproductive age, the outcome of the procedure has not yet been sufficiently high, with many patients still undergoing two or more attempts before they become pregnant and deliver an HLA-identical offspring. Testing for age-related aneuploidy, therefore, seems useful for improving the reproductive outcome after preimplantation HLA typing, which will also minimize the risk of delivering a child with chromosomal disorders and provide reassurance for patients who are concerned about their pregnancy outcomes (Rechitsky *et al*., 2006).

Despite the ethical issues involved (Edwards, 2004), preimplantation HLA typing has been performed in hundreds of cases involving affected children who require HLA-compatible stem cell transplantation, including thalassemia, Fanconi anaemia, Wiscott–Aldrich syndrome, X-linked adrenoleukodystrophy, X-linked hyper–immunoglobulin M syndrome, X-linked hypohidrotic ectodermal dysplasia with immune deficiency, incontinentia pigmenti, leukaemias and inherited and sporadic forms of Blackfan–Diamond anaemia.

PGD for chromosomal disorders

PGD for aneuploidies has been applied to cases with poor prognosis, including those with advanced reproductive age, repeated IVF failures and repeated spontaneous abortions. The potential of pre-selecting of euploid embryos for transfer is based on the well-established fact that, in cases with poor prognosis, approximately half of oocytes and embryos are chromosomally abnormal (**Table 1**). This is based on the testing of more than 20,000 oocytes using PB1 and PB2 sampling (Kuliev *et al*., 2003; Magli *et al*., 2006) and a comparable series of cleavage-stage embryos (Gianaroli *et al*., 1999, 2001; Kahraman *et al*., 2004b; Munné *et al*., 2007). As mentioned in the introduction, the accuracy of each of these approaches may be limited because oocyte testing does not pick up the errors from paternal meiosis, fertilization-related abnormalities and mitotic errors, while single biopsied blastomeres might not represent the actual karyotype of the embryo, with approximately half of abnormalities represented by mosaicism. As there is no information about the initial chromosomal set of the zygotes from which these mosaic embryos originated, the nature of mosaicism in preimplantation embryos is not known.

On the other hand, it is obvious that a significant proportion of mosaic embryos may originate from aneuploid oocytes, as suggested by a comparable prevalence of aneuploidy in oocytes and embryos with different types of chromosomal anomalies. Although there is no doubt that mosaicism is one of the major

Table 1. Occurrence of chromosomal abnormalities in human oocytes detected by fluorescent in-situ hybridization (FISH) analysis of the first and second polar bodies using specific probes for chromosomes 13, 16, 18, 21 and 22 (Kuliev *et al*., 2007).

Cycles	*Total no. of oocytes studied*	*No. of oocytes with FISH results*	*No. of normal oocytes (%)*	*No. of abnormal oocytes (%)*
3084	22,790	17,329	8871 (51.2)	8458 (48.8)

types of finding at the cleavage stage, the exact data on its rate in preimplantation development cannot be evaluated, because only a limited number of the preimplantation embryos have been fully studied. While the possibility of post-zygotic mitotic errors in cleavage-stage embryos that were originally euploid cannot be excluded, the proportion of aneuploidy and mosaicism stemming from these errors is not known, nor is the impact of these post-zygotic errors on pre- and post-implantation embryo development. Recent cytoskeletal analysis demonstrated that the abnormal spindle and chromosomal loss through anaphase lagging and congression failure found throughout preimplantation development may be responsible for the high incidence of mosaicism in preimplantation embryos (Chatzimeletiou *et al*., 2005a,b).

Based on the above data, it may be suggested that the most accurate pre-selection of embryos for transfer in PGD for aneuploidies may be performed by sequential testing of meiosis I, meiosis II and mitotic errors, through sequential PB1, PB2 and blastomere sampling. This may avoid the transfer of embryos with pre-zygotic chromosomal errors of maternal origin, which is the major source of chromosomal abnormalities in the embryo, and also the detection of paternally derived anomalies and possible mitotic errors in embryos resulting from the euploid zygotes. The accumulated data on such sequential sampling will also help to evaluate the possible differences in viability of embryos with chromosomal abnormalities of meiotic and mitotic origin. In addition, the information from both the oocyte and the embryo chromosome sets will make it possible to detect potential cases of uniparental disomy, as evidenced from the detection of normal disomic embryos that originated from trisomic oocytes. Collection of this unique information may also be useful in finding a possible explanation for at least some of the cases of imprinting disorders reported in association with assisted reproductive technology.

Although there is no doubt that PGD for chromosomal aneuploidy will potentially contribute to the pregnancy outcome for IVF cases with poor prognosis, the actual impact will depend on the completeness and accuracy of testing, which may be evaluated by reanalysis of the embryos rejected from transfer. On the other hand, the transfer of incorrectly tested abnormal embryos will lead to implantation and pregnancy failures, and therefore testing the products of conception obtained after spontaneous abortions may also be useful. No doubt, further improvement in the accuracy and completeness of chromosomal analysis will be necessary to achieve a greater impact of the procedure on clinical outcome. Although one of the developments in full karyotyping, such as comparative genomic hybridization, appears to be as yet impractical for clinical purposes (Wilton *et al*., 2003; Verlinsky and Kuliev, 2003), the recent developments in microarray technology may help a complete and accurate chromosomal analysis to be applied for PGD of aneuploidy.

Apart from a few small series, the existing experience suggests a significant impact of aneuploidy testing on the reproductive outcome for poor prognosis IVF cases (Gianaroli *et al*., 1999, 2001; Munné *et al*., 1999, 2003, 2006). Applied presently in over 20,000 IVF cycles in the effort to pre-select the embryos with highest potential to result in a pregnancy, PGD for chromosomal disorders has demonstrated a positive clinical impact through the improved implantation and pregnancy rates, reduction of spontaneous abortions and improved take-home baby rate in poor prognosis cases, including those with advanced reproductive age, repeated IVF failures and recurrent spontaneous abortions.

The lack of a positive effect of aneuploidy testing in two smaller studies (Staessen *et al*., 2004; Platteau *et al*., 2005) may be due to the potentially detrimental effect of removing two blastomeres, which definitely reduced the implantation potential of the biopsied embryos to an extent that could not be bridged even by pre-selection of aneuploidy-free embryos (Cohen *et al*., 2007). Without taking into consideration these technical details, the data were misinterpreted as the lack of impact of PGD on pregnancy outcome, although, even in the absence of differences between PGD and non-PGD groups in the above studies, they suggested a beneficial effect of pre-selection of aneuploidy-free embryos in terms of compensating for a detrimental effect of 2-cell biopsy on day 3. In the other more recent report that failed to detect a positive effect despite single blastomere biopsy, the authors excluded a few key chromosomes from testing; they also faced the very poor overall outcome of aneuploidy testing, with up to 20% failed results, which affected the appropriate pre-selection of embryos for transfer from an average of only 4.8 embryos available for testing (Mastenbroek *et al*., 2007).

Although further randomized controlled studies are still required to quantify in more detail the clinical impact of pre-selecting aneuploidy-free zygotes for embryo transfer, the positive impact of PGD is particularly obvious from the comparison of reproductive outcome in the same patients with and without PGD (Gianaroli *et al*., 2004; Verlinsky *et al*., 2005b). Implantation, spontaneous abortion and take-home baby rates were analysed before and after PGD, and appeared to be significantly improved after PGD. For example, the implantation rate prior to PGD was only 7.2%, in contrast to 34.8% after PGD, suggesting an almost five-fold improvement. As expected, there was also a significant reduction in the spontaneous abortion rate, which was 72% before and 26.9% after PGD. This accordingly contributed to the more than two-fold increase in take-home-baby rate after PGD, which was as high as 65.7% in PGD cycles compared with 27.9% without PGD.

Improvements were particularly evident in PGD for translocations, clearly the optimal solution for couples carrying translocations because of their exceptionally poor reproductive outcome even with the use of routine prenatal diagnosis (Munné 2002; Verlinsky *et al*., 2005b). A significant impact of PGD on the pregnancy outcome is obvious from the accumulated experience of more than 1000 cases, with evidence for at least a four-fold reduction of spontaneous abortions in translocation carriers compared with that before undertaking PGD. This makes PGD clearly a preferred option over traditional prenatal diagnosis for carriers of chromosomal translocations.

Conclusions

PGD has become a part of genetic practice and assisted reproduction technology and provides the option of establishing a pregnancy free from genetic and chromosomal disorders for couples at risk. Technological developments allow PGD to be performed with improved accuracy, and this has extended

the application of PGD both for avoiding the transfer of chromosomal abnormalities in IVF and for testing for an increasing number of genetic disorders and genetic variability. PGD application is no longer limited to conditions presented at birth, but also appears to be attractive for late-onset disorders with genetic predispositions. Non-disease testing is presently another practical application, which makes PGD useful for obtaining an HLA-compatible offspring to affected siblings who require stem cell transplantation therapy. This has already been applied in hundreds of cases, resulting in the successful treatment of more than dozen siblings with congenital and acquired disorders.

There is strong evidence for the clinical usefulness of PGD for IVF, involving the exclusion of aneuploid embryos from transfer in poor prognosis IVF cases, including those with advanced reproductive age, repeated IVF failures and recurrent spontaneous abortions. However, there is still a need to improve the accuracy of aneuploidy testing, to ensure that current practice can optimally benefit from the genetic pre-selection of embryos with the highest developmental potential as an alternative to the currently practiced pre-selection of embryos for transfer using morphological criteria.

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