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An update of preimplantation genetic diagnosis in gene diseases, chromosomal translocation, and aneuploidy screening

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Preimplantation genetic diagnosis (PGD) is gradually widely used in prevention of gene diseases and chromosomal abnormalities. Much improvement has been achieved in biopsy technique and molecular diagnosis. Blastocyst biopsy can increase diagnostic accuracy and reduce allele dropout. It is cost-effective and currently plays an important role. Whole genome amplification permits subsequent individual detection of multiple gene loci and screening all 23 pairs of chromosomes. For PGD of chromosomal translocation, fluorescence *in-situ* hybridization (FISH) is traditionally used, but with technical difficulty. Array comparative genomic hybridization (CGH) can detect translocation and 23 pairs of chromosomes that may replace FISH. Single nucleotide polymorphisms array with haplotyping can further distinguish between normal chromosomes and balanced translocation. PGD may shorten time to conceive and reduce miscarriage for patients with chromosomal translocation. PGD has a potential value for mitochondrial diseases. Preimplantation genetic haplotyping has been applied for unknown mutation sites of single gene disease. Preimplantation genetic screening (PGS) using limited FISH probes in the cleavage-stage embryo did not increase live birth rates for patients with advanced maternal age, unexplained recurrent abortions, and repeated implantation failure. Polar body and blastocyst biopsy may circumvent the problem of mosaicism. PGS using blastocyst biopsy and array CGH is encouraging and merit further studies. Cryopreservation of biopsied blastocysts instead of fresh transfer permits sufficient time for transportation and genetic analysis. Cryopreservation of embryos may avoid ovarian hyperstimulation syndrome and possible suboptimal endometrium.

Keywords: Array Comparative Genomic Hybridization; Preimplantation Genetic Diagnosis; Preimplantation Genetic Screening; Single Nucleotide Polymorphisms Array; Vitrification; Whole Genome Amplification; Human

Introduction

Preimplantation genetic diagnosis (PGD) was first successfully performed in humans for prevention of X-linked disease in 1990 [1]. It gains a gradually important role in the prevention for Mendelian hereditary diseases and unbalanced chromosomal translocation. PGD provides an alternative of prenatal diagnosis with selective termination of pregnancy for couples accompanying a high risk of affected offspring. More indications have been applied as aneuploidy screening, human leukocyte antigen (HLA) typing, adult-onset Mendelian diseases, cancer predisposition syndromes and mitochondrial diseases [2,3]. Significant advancement has been attained in biopsy technique and molecular diagnosis. Vitrification of embryo after blastocyst biopsy permits sufficient time for shipment of specimen and diagnosis in the orderly fashion [4]. In this update, we will review indications, present approaches to retrieve cells for PGD, diagnostic

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technology, accuracy and possible misdiagnosis in details.

PGD for single gene disease and three different biopsy approaches

In principle, all monogenic disorders for which prenatal diagnosis is available can be examined by PGD as well. Many diseases have been successfully applied including thalassemia, cystic fibrosis, hemophilia, spinal muscular atrophy, neurofibromatosis, and congenital deafness, etc. [1-7]. There are three potential sources of embryonic genetic material for PGD, including polar bodies from oocytes, blastomeres from cleavage-stage embryos, and trophectoderm cells from blastocysts [8,9]. The polar bodies can be used for the indirect genetic analysis of the oocyte. The disadvantage is that it does not permit the analysis of the paternal genetic material. Since beginning, cleavage-stage biopsy with aspiration of one blastomere is the most common approach for PGD. In the recent years, blastocyst biopsy is gradually widely used. Both of them allow analysis of maternal and paternal origin.

PCR-based tests using single cell have possibility of ADO

Polymerase chain reaction (PCR) of single cells is vulnerable to contamination and allele dropout (ADO). ADO occurs when one of the two alleles fails to be amplified and only the other allele is amplified. In autosomal-dominant disorders, ADO of the affected allele might lead to the transfer of an affected embryo. For autosomal recessive conditions, ADO will not cause serious misdiagnosis [7], but the number of embryos available for transfer would reduce and this may result in a decreased pregnancy rate. The data of ADO in direct amplification of gene locus ranges from 5% to 33% [10,11]. The ADO rates in whole genome amplification (WGA) are from 5% to 31% [12,13], which are comparable to those obtained by direct amplification of gene locus. The incidence of ADO may depend on the cell number analyzed, the genes tested, and the lysis conditions, the PCR conditions, or the method of WGA [14]. To reduce the ADO rate below 10% would be imperative [15].

Polymorphic markers are helpful to find ADO that prevents misdiagnosis

Polymorphic markers with short tandem repeats (STR) or single nucleotide polymorphisms (SNP) close to the mutated gene have been used for linkage analysis to provide an additional confirmation of genotyping and an evidence of ADO [16,17]. STR markers also help to detect contamination of exogenous DNA with different STR sizes. Sufficient numbers (at least three) of linked markers are recommended to be tested together with the causative gene [15].

Blastocyst biopsy may increase diagnostic accuracy and reduce ADO. It is cost-effective and may replace cleavage-stage biopsy

Blastocyst biopsy providing more cells than the cleavage-stage biopsy (5-10 cells vs. 1-2 cells) for genetic analysis may potentially reduce the risk of amplification failure and ADO [18]. Kokkali et al. [18] prospectively compared genotyping success and implantation rates in PGD cycles after biopsy at the blastocyst versus the cleavage stage. They found that the genotyping success rate was significantly higher in the blastocyst group (94%) than in the cleavage-stage group (75%). The implantation and pregnancy rates were not statistically different. With the higher genotyping success rates, trophectoderm biopsy may result in relatively more embryos available for transfer. Trophectoderm biopsy would be relatively cost-effective and less labor-intensive, because only embryos competently developing to the blastocyst stage are biopsied.

Blastocyst biopsy plus vitrification permits sufficient time for diagnosis, and may also avoid suboptimal endometrium

Embryo biopsy and fresh embryo transfer are traditionally performed in the PGD cycle. However, prior to embryo transfer, the time allowed for shipment of the specimens to the reference laboratory and performance of molecular diagnosis is limited, especially after blastocyst biopsy. Vitrification of blastocysts has been demonstrated to achieve high survival rate and pregnancy rate [19]. The strategy of PGD for monogenic diseases using blastocyst biopsy, vitrification, WGA accompanied by double confirmatory genotypings, and thawed embryo transfer have been successfully performed [4]. In addition, cryopreservation of embryos may be beneficial for patients of high responder to circumvent risk of ovarian hyperstimulation syndrome and possible suboptimal endometrium [20-22].

PGD for adult-onset autosomal diseases and cancer predisposition syndromes

PGD to exclude transmission of an autosomal dominant adult-onset disease would be more acceptable to at-risk families than the pregnancy termination after prenatal diagnosis. Huntington disease or other neurodegenerative diseases have been successfully applied [23]. PGD has also been performed for familial cancer syndromes including BRCA1, multiple endocrine neoplasia, familial adenomatous polyposis, Li-Fraumeni syndrome, retinoblastoma and von Hippel-Lin-

dau syndrome [24]. For hereditary breast and ovarian cancers caused by mutations in BRCA 1 and 2, the life-time risk of breast cancer for a female carrier may be as high as 85%, her risk of ovarian cancer as high as 60% [25].

PGH for unknown mutation sites of single gene disease

Preimplantation genetic haplotyping (PGH) can detect any mapped single gene diseases with unknown mutation sites [26]. It requires a comprehensive study of the pedigree with at least one affected family member and finding multiple informative linked markers for the mutated genes before PGH can be performed. PGH has been successfully applied to monogenic diseases following WGA with multiple displacement amplification (MDA) [27,28]. PGH after WGA permits examinations of a large number of polymorphic markers.

PGD with HLA haplotyping and stem cell transplantation

In families having a diseased child with recessive genetic disorders such as Fanconi anemia or thalassemia, the couple undergoing PGD and HLA typing may not only avoid another genetically diseased child but also have a HLA-compatible sibling of the diseased child. The likelihood of a genetically normal and HLA-compatible embryo is 3 in 16. Therefore, perhaps several treatment cycles would be needed before a pregnancy can be achieved. After birth of the neonate, umbilical cord blood can be collected and hematopoietic stem cell transplantation can be applied to cure the diseased child [5,29]. The positive ethical background has been recently well discussed [30].

A potential, but uncertain, value of PGD for mitochondrial disease with unknown nuclear gene defects

PGD provides a reasonable option for mitochondrial disease with nuclear gene defects, like the treatment of autosomal recessive disease [2]. For the patients with unknown nuclear gene defects, the heredity of mitochondrial disease is uncertain. A woman carrying a mitochondrial DNA (mtDNA) mutation could be heteroplasmic and could transmit a various amount of mutation to her offspring. The phenotype can not be accurately predicted. In this situation, like prenatal diagnosis, the value of PGD would be doubtful because no definite disease-free diagnosis can be achieved. However, by transferring low mutant load of embryos, PGD may be performed to reduce the risk of disease for women with a high mutation load [31]. Technically, the high copy number of mtDNA makes the analysis feasible with less amplification failure and ADO [2]. But, the heteroplasmic characteristics may raise a question concerning the representation of biopsied samples for the whole embryo. For embryo transfer, a cut-off point should be set regarding the mutant load of embryos. Preferential transfer of male embryos with low mutant load could be a morally acceptable way of avoiding transgenerational risks [32].

WGA permits individual detection of multiple gene loci and screening all 23 pairs of chromosomes

Single cell or few cells obtained for PGD contains very limited DNA material. In order to detect mutated gene and polymorphic markers, multiplex PCR was traditionally used. It is critical to design compatible multiple primer sequences and conditions when several PCR reactions may interfere mutually in a tube. If one of the PCR reactions failed, the detection procedures can not be repeated. The utilization of WGA to produce a DNA library could provide sufficient DNA templates for multiple independent examinations of gene mutations and polymorphic markers as well as repeating confirmations for an uncertain result. Different genotyping methods can also be used for double confirmations of diagnosis [4]. The WGA products also allow detecting chromosomal translocation and screening all 23 pairs of chromosomes.

A few WGA methods have been used in PGD [5,33]. Primer extension pre-amplification (PEP) using a random mixture of 15-base oligonucleotides was estimated to amplify 91% genome of a cell. However, the microsatellite loci used for fingerprinting were not accurately amplified. OmniPlex converts randomly fragmented genomic DNA into a library, and has an amplification of $>99.8\%$ genome achieving a SNP call rate of $>98\%$. MDA using bacteriophage Phi29 DNA polymerase and random hexamer primers is recently developed for WGA with unbiased amplification [13,34]. MDA is a non-PCR-based method that may prevent generation of relatively short DNA fragments and avoid possibility of mutation introduction [33]. A few clinical applications of MDA for PGD have been reported, such as for Marfan syndrome, Duchenne muscular dystrophy, cystic fibrosis, β-thalassemia, and Hungtington chorea, and neurofibromatosis type 1 [4]. The other WGA methods including linker adaptor and GenomePlex have been investigated [35,36]. The advantages of each WGA method in PGD deserve further studies.

PGD provides an alternative to achieve normal pregnancy for patients with chromosomal translocation

It is estimated that 1 in 625 individuals carries a balanced chromosomal translocation. In couples with recurrent miscarriage, the inci-

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dence of either of the couple being a carrier of a structural chromosome abnormality is approximately 4-5%, mainly including reciprocal translocations and Robertsonian translocations [37]. In the case of reciprocal translocation, at meiosis I during gametogenesis, the two pairs of homologous chromosomes associate at the pachytene stage to form a quadrivalent with matching of homologous segments. Several modes of segregation including 2:2 alternate, adjacent-1, adjacent-2, 3:1 and 4:0 may occur. At meiosis II, anaphase non-disjunction may also happen. The theoretical chance of producing normal or balanced gametes is 4 of 32 for reciprocal translocation, and it is 4 of 16 for Robertsonian translocations. However, the actual percentage depends on several factors, including which chromosomes involved, the breakpoints, and the sex of the carrier [38]. The carriers of balanced chromosomal translocation are at increased risk for infertility, pregnancy loss, and offspring with congenital abnormalities and mental retardation as a result of unbalanced segregation. PGD provides an option to exclude the unbalanced embryos.

FISH is traditionally used for PGD of chromosomal translocation, but with technical difficulty

Initial research of chromosomal translocation using Fluorescence *in-situ* hybridization (FISH) involved painting probes for metaphase chromosomes of polar bodies. A major shortcoming of this method is that only translocations of the female can be examined. Then, probes that spanned or flanked the translocation breakpoints were used for the interphase nucleus of a blastomere from cleavage-stage embryos. It can distinguish normal embryos from embryos of balanced and unbalanced translocation. But this methodology was restricted by the cost and the time needed to make specific probes. The subtelomere probes in combination with centromeric probes are commonly used that obviated the need for specific probe development for each rearrangement [38,39]. But this strategy will not differentiate between normal and balanced embryos. The FISH method is technically difficult on fixation of blastomeres. Several drawbacks may be encountered including hybridization failure, signal overlap, and splitting that can affect the accuracy of the interpretation [40]. FISH analysis may offer a positive predictive value of 83% and negative predictive value of 81% [41]. Wrong results may eliminate normal/balanced embryos for transfer, or lead to transfer of abnormal embryos.

PCR-based PGD for chromosomal translocation avoids difficulty of cell fixation

The PCR-based PGD protocol for translocations has the potential to overcome several inherent limitations of FISH-based tests [17]. They are not dependent on cell fixation onto a microscope slide, a critical step that requires skill and experience, with the potential to increase the percentage of embryos with a positive result. It is easier to train staff in, and monitor, the placing of single cells into PCR tubes than teaching the fixation. It has potential improvements in terms of test performance, automation, turnaround time, sensitivity, and reliability. Both FISH and PCR-based methods allow identification of aneuploidies simultaneously, but only for a limited number of chromosomes. The array comparative genomic hybridization (CGH) after WGA has the above advantages and can detect all 24 chromosomes.

Array CGH detects translocation and 23 pairs of chromosomes and may replace FISH for PGD of chromosomal translocation

Array CGH permits visualization of all 46 interphase chromosomes. The tested sample genome is first amplified through WGA and labeled with one color. The normal reference sample is also amplified and labeled with another color. These samples are used as probes to hybridize onto normal metaphase chromosomal plates. With image-processing software, unbalanced structural differences between the normal reference and test sample can be shown [42]. Array CGH provides the added benefit of simultaneous aneuploidy screening of all 24 chromosomes [43,44]. Another advantage of array CGH is that it does not require preclinical validation before each IVF cycle, which is required for FISH. This avoids postponement of IVF treatment. Fiorentino et al. [44] reported 28 cycles of PGD at the cleavage embryo for chromosomal translocations. A high percentage of embryos (93%) were successfully diagnosed. Embryos suitable for transfer were in 60% of started cycles. A 70% of pregnancy rate per transfer cycle was achieved. Array CGH merits further validation in the blastocyst biopsy.

SNP microarray can distinguish between normal chromosomes and balanced translocation

SNPs account for most gene variants found in humans and can be used as markers for genome-wide association study. SNP array analysis after WGA not only explores chromosomal aberrations but also provides information of haplotypes [45]. It can distinguish between normal and balanced chromosomes in embryos from translocation carriers. In addition, SNP array can also screen aneuploidy of all 24 chromosomes. Treff et al. [46] reported successful application of SNP array for PGD to distinguish between normal and balanced chromosomes in embryos from a translocation carrier. This may provide an important option for these patients to prevent their offspring from facing the same fertility problem.

For patients of balanced chromosomal translocation, the live birth rates are similar between PGD and natural conception

Franssen et al. [37] performed a systematic review and found that after natural conception, live birth rate per couple varied between 33 and 60% (median 55%). After PGD, live birth rate per couple varied between 0 and 100% (median 31%). It has been reported that 15-26% of the embryos were transferable in patients of reciprocal translocations, confirming the high level of chromosomally abnormal embryos [38]. This might explain why the results of PGD in some couples are relatively low [47]. Therefore, there are insufficient data indicating that PGD improves the live birth rate in couples with recurrent miscarriage carrying a structural chromosome abnormality.

PGD may shorten time to conceive and reduce miscarriage for patients of balanced chromosomal translocation

For patients with chromosomal translocation and repeated miscarriage, PGD may reduce time to get pregnancy. In the recent two reports, the cumulative pregnancy rate using PGD was 57-87%, involving an average of 1.2-1.4 cycles [48,49]. The short time (<4 months) undergoing PGD achieves pregnancy in contrast with the much longer interval (mean, 4-6 years) for natural conception. Pregnancy loss rate was significantly reduced to 13% post-PGD compared with 88. 5% in previous non-PGD pregnancies. This is close to a sevenfold reduction in loss rate.

Costs and benefits of PGD in chromosomal translocation should be considered for each couple

PGD is an option when the couple with balanced translocation has also a fertility problem. For those couple without fertility difficulty, the costs and benefits should be considered in light of the limited change in live birth rate. PGD may shorten the time to achieve normal pregnancy and reduce miscarriage rate. The pregnancy rate is higher for selected patients with younger age and many good quality embryos available. But, the pregnancy rate would be lower for patient with older age and few embryos available. The possible treatment options should be provided and discussed with the couple.

The rationales of PGS for advanced maternal age, idiopathic repeated pregnancy loss, recurrent aneuploidy, or repeated implantation failure

Pregnancy rates and live birth rates decline with increasing maternal age. Miscarriage rates increases also. The primary reason is high embryonic loss due to aneuploidy. It has been found that 50% of morphologically normal embryos in women >35 years old are chromosomally abnormal [50]. More than 50% of first-trimester spontaneous abortions are chromosomal aneuploidy. Hence, it could be effective to increase live birth rates by performing PGS and transferring euploid embryos for patients of advanced maternal age, repeated implantation failure, and idiopathic recurrent pregnancy loss. It may be applied in single embryo transfer. It is supposed to improve implantation rates, reduce miscarriages and trisomic offspring, and ultimately lead to an increase in live birth rates [51,52]. However, aneuploidy is not the absolute cause of implantation failure or idiopathic recurrent pregnancy loss. The cost and benefits of PGS should be considered for individual patients.

PGS using limited FISH probes in the cleavage-stage embryos is not effective

Since the late 1990s, favorable ongoing pregnancy rates for advanced maternal age were achieved in initial studies using FISH-based PGS on cleavage-stage embryos [53]. However, the recent ten randomized controlled trials have shown that PGS does not increase live birth rates, although these studies are all confounded by limited power. Meta-analysis of these trials shows a statistically significant reduction of ongoing pregnancies after PGS (13% after PGS, vs. 21% in the control group) [53]. For unexplained recurrent miscarriage, there were no randomized controlled trials on this topic. A systematic review was to assess live birth rates and miscarriage rates after PGS or natural conception [54]. From the limited data, a similar live birth rate is reported for PGS and natural conception (35% and 42%, respectively). The miscarriage rate for the PGS group (9%) appears to be lower than in the natural conception group (28%). For repeated implantation failures, the existing data of PGS are also limited and inconsistent. Some studies are suggestive of increased pregnancy rates for younger women (average age, 30-33 years), while others did not verify any advantage [55]. Overall, the present data are not sufficient to support the use of PGS and FISH at the cleavage-stage embryos to improve the live birth rates in patients with advanced maternal age, recurrent pregnancy loss, or repeated implantation failure.

Polar body and blastocyst biopsy may circumvent the problem of mosaicism of the cleavage-stage embryo biopsy

Two major factors may explain the negative result of PGS and FISH on day 3 embryos, including chromosomal mosaicism and insufficient number of chromosomes tested [56]. The tested blastomere

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may not be representative for the whole embryo owing to mosaicism or the possibility of self-correction of aneuploidy within the embryo. As mosaicism is not present at the oocyte, it seems to be preferable to opt for polar body biopsy. The disadvantage of polar bodies is that only the maternal aneuploidies can be studied. However, the vast majority (more than 90%) of human aneuploidies is maternal origin. Testing the polar body is ethically acceptable in countries that do not allow testing of embryos. PGS at the blastocyst stages may provide more accurate information for chromosome abnormalities than at the cleavage stage. Although blastocysts may also display mosaicism, several cells can be biopsied at this stage that is more representative for the whole embryo [57].

PGS using blastocyst biopsy or polar body biopsy and array CGH may replace cleavage-stage biopsy and limited FISH assay

Initial results for patients of advanced maternal age with repeated implantation failure receiving blastocyst biopsy and array CGH for PGS were encouraging [56]. Fragouli et al. [56] found that implantation and pregnancy rates for the patients with polar body biopsy were 11% and 21%, respectively, whereas for patients receiving blastocyst biopsy they were 58% and 69%. Blastocyst analysis was associated with high pregnancy rates, suggesting that comprehensive chromosome screening may assist these patients in achieving pregnancies. Blastocyst or polar bodies biopsy using array CGH are recommended for randomized controlled trials for women of advanced maternal age, idiopathic recurrent abortions or repeated aneuploidy, and repeated implantation failure.

Misdiagnosis may occur in PGD and prenatal confirmation is recommended

Some misdiagnoses of PGD had been reported in the literature [9, 40]. The causes of misdiagnosis may include maternal or paternal contamination, ADO, and chromosomal mosaicism [40]. In face of the risk of misdiagnoses, prenatal diagnosis is recommended to confirm the PGD result when a pregnancy is achieved.

PGD babies do not increase for congenital abnormalities

There are some cohort studies concerning the pediatric follow-up of PGD babies. Liebaers et al. [58] reported a thorough, systematic study of PGD offspring judged on the basis of a physical examination 2 months after birth. Anomalies in PGD babies, compared to a previously reported cohort study of ICSI offspring, structural malformations were found in 2.13% for PGD and 3.38% for ICSI. Nekkebroeck et al. [59] found similar mental and psychomotor developmental outcomes at age 2 when compared with children conceived after ICSI and natural conception. PGD babies do not appear to be increased in anomalies.

Ethic aspect of PGD

PGD is gradually widely acceptable as an alternative to prevent the birth of a child with a hereditary disease including single gene disorder and chromosomal rearrangements. PGD for autosomal dominant late-onset disorders, cancer predisposition syndrome, PGD/HLA typing, and mtDNA mutations is more controversial. The experienced geneticist and infertility doctor should provide comprehensive counseling to the couple and explain the theoretical percentage of unaffected embryos. The severity of the disease, the age of onset of the disease, and the penetrance of the gene mutation are discussed. The odds of a misdiagnosis inherent to PCR or FISH are explained, and subsequent genotype confirmation of the pregnancy using conventional prenatal diagnosis is indicated. The cost, risk, and benefits should be well-explained. The decision would be determined by the couples.

Conclusion

PGD gains a gradually important role in the prevention of Mendelian hereditary diseases and unbalanced chromosomal translocation. More indications have been applied as aneuploidy screening, HLA typing, adult-onset autosomal disease, cancer predisposition syndrome and mitochondrial disease. Polymorphic markers are essential to find ADO that prevents misdiagnosis. Blastocyst biopsy increases diagnostic accuracy that may replace the cleavage-stage biopsy. PGD may shorten time to conceive and reduce miscarriage for patients with chromosomal translocation. PGS using blastocyst biopsy and array CGH is encouraging. Cryopreservation of biopsied blastocysts permits sufficient time for molecular diagnosis. That may also circumvent risk of ovarian hyperstimulation syndrome and possible suboptimal endometrium. Array CGH can detect translocation and 23 pairs of chromosomes and may replace FISH for PGD of chromosomal translocation and PGS. PGD can currently offer with a high accuracy of 95-99%, and may result in a pregnancy rate of 20-60%, depending on indications and age.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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