

Preimplantation testing: Transition from genetic to genomic diagnosis

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Abstract

Preimplantation genetic testing refers to the procedure to determine the genetic status of embryos formed by *in vitro* fertilization (IVF) prior to initiating a pregnancy. Traditional genetic methods for preimplantation genetic diagnosis (PGD) examine distinct parts of an individual genome, require the development of a custom assay for every patient family, and are time consuming and inefficient. In the last decade technologies for whole-genome amplification (WGA) from single cells have led to innovative strategies for preimplantation testing. Applications of WGA technology can lead to a universal approach that uses single-nucleotide polymorphisms (SNPs) and mutations across the entire genome for the analysis. Single-cell WGA by multiple displacement amplification has enabled a linkage approach to PGD known as "preimplantation genetic haplotyping", as well as microarray-based techniques for preimplantation diagnosis. The use of microarrays in preimplantation diagnosis has provided genome-wide testing for gains or losses of single chromosomes (aneuploidies) or chromosomal segments. Properly designed randomized controlled trials are, however, needed to determine whether these new technologies improve IVF outcomes by increasing implantation rates and decreasing mis-

carriage rates. In genotype analysis of single cells, allele dropout occurs frequently at heterozygous loci. Preimplantation testing of multiple cells biopsied from blastocysts, however, can reduce allele dropout rates and increase the accuracy of genotyping, but it allows less time for PGD. Future development of fast SNP microarrays will enable a universal preimplantation testing for aneuploidies, single-gene disorders and unbalanced translocations within the time frame of an IVF cycle.

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INTRODUCTION

As an alternative to prenatal diagnosis, preimplantation genetic diagnosis (PGD) was initiated in the late 1980s to enable couples at risk of transmitting inherited genetic disorders to give birth to healthy children. Genetic disorders can be an inherited genetic condition, or due to an abnormal number or structure of chromosomes. PGD determines the embryo's genotype allowing couples to select embryos unaffected with genetic disorders prior to initiating a pregnancy, whereas prenatal diagnosis often leads to selective termination of an affected pregnancy^[1-4]. Since there are more than 6000 single gene disorders af-

fecting approximately 1 in 300 live-births^[5], the medical need for PGD service is significant.

In the early 1990's, fluorescent *in situ* hybridization (FISH) was used by Handyside's group to assess an embryo's karyotype for aneuploidies^[6] and by Munne's team for gender determination^[7]. FISH was also used in pre-implantation testing for chromosomal translocations^[8]. Since cytogenetic abnormalities cause approximately one quarter of miscarriages and stillbirths^[9], the medical need for aneuploidy testing is also significant.

PGD BY DIRECT MUTATION DETECTION

For PGD, the most widely used stage for testing has been the 8-cell cleavage-stage embryo, from which one or two blastomere(s) are removed for testing^[10]. The first techniques employed in PGD used direct mutation analysis after amplification of genomic DNA from single blastomeres by PCR. Gene mutations were often detected by analysis of PCR products using restriction enzyme cleavage, also known as restriction fragment length polymorphism (RFLP)^[11-13], or by minisequencing^[14].

In genotype analysis of single cells, allele dropout (ADO) occurs frequently at heterozygous loci^[15]. Misdiagnosis due to ADO can be minimized by haplotype analysis of embryos with tightly linked short tandem repeat (STR) markers in addition to direct mutation detection^[16].

PGD BY WHOLE-GENOME AMPLIFICATION AND LINKAGE APPROACH

Novel technologies for whole-genome amplification (WGA) from single cells provide a universal first step for generating many copies of the entire human genome, and have led to innovated strategies for preimplantation testing^[17]. Newer technologies for single-cell WGA include multiple displacement amplification (MDA)^[17-19] and the single-cell WGA system of Rubicon Genomics (Ann Arbor, Michigan). Rubicon's WGA technology is based on the construction of a PCR amplifiable library^[20], whereas MDA is a non-PCR based isothermal method for WGA^[21]. Application of a commercial MDA kit (Molecular Staging, Inc., New Haven, CT) for the amplification of single cells resulted in high ADO rates for genotyping^[17]. Since the application of MDA for single cells is not a standard application supported by the kit manufacturers (GE Healthcare or Qiagen), improvements have been made to lower ADO rates by optimizing the procedures of single-cell preparation and MDA reaction^[19,22].

Indeed, the use of single-cell WGA by MDA has enabled a novel approach known as "preimplantation genetic haplotyping" (PGH) for PGD of single-gene disorders, which is based on linkage analysis with STR markers^[23]. The first round of PGH is WGA of single blastomeres by MDA. The second round involves geno-

type analysis with linked STRs using whole-genome amplified DNA as template. A comparison of sizes and genotypes of STRs among family members allows inferences of the haplotypes for parents and an affected child. Unaffected embryos can then be identified by comparing their haplotypes to those of the parents and affected sibling^[23-25].

The main advantage of PGH for single-gene disorders is that it skips direct mutational analysis of embryos, but determines the haplotypes of an embryo with a panel of informative STRs for the disease locus and its flanking regions. STR haplotyping had also been applied to PGD for HLA matching^[26-28]. In fact, PGH can be applied to families whose exact mutational bases are unidentified (Eduardo Lau, Marleen Janson and David Bick, Medical College of Wisconsin, Milwaukee, Wisconsin, unpublished data).

HIGH-THROUGHPUT PREIMPLANTATION TESTING BY DNA MICROARRAYS

Although PGH is a generalized method for PGD of single-gene disorders, the development of custom assays for individual disorders and patient families is time consuming and inefficient. In fact, custom PGD assays have been developed for less than 10% of the known single-gene disorders after 21 years^[3]. Preimplantation testing by the FISH technique usually examines only a few chromosomes (e.g., 13, 18, 21, X, Y, 15, 16 and 22) that are responsible for common aneuploidies in liveborns and the most common early miscarriages, and this requires two rounds of hybridization^[3].

While traditional PGD scrutinizes the structure and composition of single genes or chromosomes, a genomic approach that combines robust WGA of single blastomeres with high-throughput microarrays interrogates the complete genome of an embryo to identify single nucleotide polymorphisms (SNPs), mutations and aneuploidies. Two types of DNA microarrays, namely, array-comparative genomic hybridization (aCGH)^[29-32] and SNP microarrays^[33,34], have been used in preimplantation testing. WGA of single blastomeres or several trophectoderm cells provides sufficient amplified DNA for either type of microarray analysis.

aCGH

aCGH measures the hybridization signals from the amplified DNA of an embryo and a control genomic DNA on a matrix of known DNA sequences (the probes) immobilized on a glass slide. This technology is able to detect genomic copy number variations throughout the 24 chromosomes at high resolution^[29].

In the past, it was a technical challenge to complete preimplantation testing by aCGH within the time frame of an *in vitro* fertilization (IVF) cycle. The procedure of aCGH was unable to meet the 2-d turn-around-time of PGD, and thus embryo freezing was required. Recent development of oligonucleotide-based aCGH with rapid

procedures allowing fast results have established aCGH as an alternative to FISH for preimplantation screening of aneuploidies, unbalanced translocations and complex karyotypes with multiple rearrangements^[29-32].

Since aCGH interrogates every chromosome and reveals submicroscopic chromosomal duplications and deletions, it is able to identify chromosome anomalies that a standard 12-chromosome FISH might fail to detect. However, aCGH often fails to detect balanced rearrangements or triploidy^[31,35,36].

SNP microarrays

A SNP microarray contains immobilized DNA sequences (the target) and detects SNPs within a genome at a high resolution. A single SNP microarray which generates predictive SNP haplotypes for the entire genome has been used in preimplantation testing for both aneuploidies and single-gene disorders^[33,34]. The SNP genotypes of two parents and an affected child define maternal and paternal haplotypes at a gene of interest. Linkage then establishes the genetic risk for an embryo based on its combination of parental haplotypes. Handyside *et al*^[33] employed SNP genotype data generated by SNP microarrays to create “karyomaps” that represent the parental haplotypes and points of recombination along a child’s chromosomes. Johnson *et al*^[34] showed that integrating SNP genotype data with gene copy number improved the quality of analysis for those “noisy” data derived from single-cell WGA. When fast SNP microarrays are available, these will be the predominant platforms for preimplantation testing for both aneuploidies and single-gene disorders.

CAN PREIMPLANTATION DIAGNOSIS BY DNA MICROARRAYS IMPROVE IVF OUTCOMES?

Since earlier techniques that interrogate 8 to 12 chromosomes by FISH failed to demonstrate a benefit of improving IVF outcomes, there remains considerable controversy regarding the need for and the ability of PGD to increase implantation rates of IVF^[37-40]. Microarrays which are able to interrogate every chromosome and identify submicroscopic chromosomal duplications and deletions may, however, increase IVF outcomes^[41].

Numerous studies suggest that early embryos apparently have a high incidence of aneuploidy and mosaicism of blastomeres^[42,43]. During development toward the blastocyst stage, aneuploid and mosaic embryos often undergo “self-correction” and lead to normal outcomes following an aneuploid finding based on a single blastomere^[44]. Therefore, there is a concern about the value and accuracy of preimplantation aneuploidy testing at the cleavage stage, since both false negative and false positive test results may be expected from these blastomeres.

Although microarrays are more consistent than FISH technique for detecting aneuploidy, both FISH and mi-

croarrays may be limited in predicting IVF outcomes in view of the high incidence of aneuploidy and mosaicism of blastomeres. Properly designed randomized controlled trials are needed to evaluate if microarray-based PGD improves IVF outcomes by increasing implantation rates and decreasing miscarriage rates^[36].

Even though it is possible to avoid the transfer of aneuploid embryos that will eventually fail to develop using PGD, other inherited factors also determine the developmental potential of an embryo. For instance, developmental events occurring at the two- to four-cell stage are known to determine whether an embryo is able to reach to the blastocyst stage^[45].

CAN NEXT-GENERATION DNA SEQUENCING BE APPLIED TO PREIMPLANTATION TESTING?

Whole-genome sequencing, which is capable of detecting SNPs and mutations at the finest resolution, could be used in preimplantation aneuploidy testing and PGD for single-gene disorders by SNP haplotyping. Compared to microarrays, PGD by sequencing is more expensive and technically demanding. Although the reagent cost for whole-genome or exome sequencing continues to fall and the speed of sequencing is greatly accelerated, the computational time needs to be tremendously shortened in order to complete sequence analysis within the 2-d timeframe of preimplantation testing.

Single-molecule sequencing (the 3rd generation sequencing platforms, e.g., Pacific Biosciences’ RS fluorescent sequencer) is more rapid and cost effective than previous “massively parallel sequencing” technologies (e.g., Roche 454, Illumina HiSeq2000 and Life Technologies’ SOLiD™ platforms), but still requires approximately 1 µg of genomic DNA template to obtain the complete human genome sequence.

It is a technical challenge to generate sufficient amounts of whole-genome amplified DNA with high fidelity for complete genome sequencing from a single human cell^[46], since commercial MDA kits are optimized for greater than 10 ng input genomic DNA. Our laboratory has, however, developed a WGA protocol by MDA that generated approximately 100 ng template-dependent amplified DNA (equivalent to about 15 000 genomes) with high fidelity from a single human blastomere in 4 h^[19]. Excessive WGA by current MDA technology generates amplified DNA of low fidelity.

In order to generate complete genome sequences from a single human cell, the analytical sensitivity of sequencing method needs to be improved. Alternatively, PGD by targeted sequencing of the disease gene may require less amount of amplified DNA template.

For PGD, larger amounts of whole-genome amplified DNA can be generated from several trophoblast cells biopsied from blastocysts on day 5 after fertilization^[47,48]. By genotyping multiple cells, the ADO rates are also

lower and the accuracy of SNP genotyping increases^[49,50]. A drawback of blastocyst testing, however, is that it allows less time for PGD, and thus the embryos may need to be frozen for transfer in the next fertilization cycle if test results are not obtained within a day^[48].

ETHICAL CONCERNS OF PREIMPLANTATION TESTING

Confidentiality of genomic data

Genomic technologies acquire the nucleotide sequence and SNPs of the entire genome rather than distinct parts of an individual genome, and thus obtain increased knowledge about genotypes and diseases. The scientific benefits of whole genome analysis are, however, accompanied by legal and ethical concerns. Informed consent and legislation are needed to protect these genomic data, as well as the privacy and confidentiality of patients' families^[51,52].

Non-medical use of preimplantation testing

While preimplantation testing was initiated to help patient families at risk for severe genetic diseases avoid the transmission of these medical conditions^[1,2], non-medical use of PGD presents some ethical concerns^[53]. For instance, non-medical use of PGD for gender selection for family balancing is a controversial issue. Although there is no broad cultural preference for male or female offspring in the U.S., there is a preference of males in some countries. In fact, non-medical use of PGD for family balancing is prohibited in many countries^[54], mainly because it could disrupt the sex ratio of the population.

FUTURE PROSPECTS FOR PREIMPLANTATION TESTING

Universal access to preimplantation testing

Since the majority of fertility centers in the United States do not have the facilities and expertise for PGD, preimplantation testing is often conducted *via* "mail order" service by sending biopsied blastomeres to a few nationwide preimplantation testing providers (Strawn EY, MD, Medical College of Wisconsin, Milwaukee, Wisconsin, unpublished data). That would obviate the high cost of setting up dedicated laboratories for PGD, and developing PGD assays for a small market.

In the United States, insurance coverage of IVF is only mandated by certain states, and the cost of preimplantation testing may not be covered by health insurance. The medical community has begun to address the medical and economic implications of implementing a national PGD program to help couples who are carriers of severe single-gene disorders, such as cystic fibrosis^[55,56]. Economic and medical considerations favor a universal and affordable access to PGD and IVF services for carrier couples of the most common and severe single-gene disorders, but cannot afford it^[57].

Besides cystic fibrosis for Caucasians of Northern European ancestry, carrier couples of common severe single-gene disorders that may benefit from PGD include fragile X syndrome^[58], Duchenne muscular dystrophy^[59], Huntington diseases^[60], hemoglobinopathies such as α - and β -thalassemia^[61-63] for people of Mediterranean and Southeast Asian heritage, spinal muscular atrophy^[12,13,64] and type 1 myotonic dystrophy^[65].

Potential applications of other "omics"

Based on the hypothesis that "viable" (developmentally competent) embryos have distinctly different gene expression profile to "non-viable" embryos, it may be feasible to identify developmentally competent embryos for transfer and thus improve implantation rates and IVF outcomes by analyzing the transcriptome profile of early embryos using cDNA microarray^[66,67] or RNA-seq analysis^[68].

Other "omics" such as proteomic^[69,70] and metabolomic technologies^[71,72] might alternatively be used to analyze the embryos and culture media, respectively, to identify developmentally competent embryos for transfer.

CONCLUSION

Severe genetic disorders are debilitating and incurable, and the lifetime medical fees for treating these sick children can be very high. The patient family and the society at large each have an interest in avoiding the birth of a child with such a disease. Preimplantation testing provides that option in a cost-effective manner, without resort to the termination of an affected pregnancy. In contrast to preimplantation genetic and cytogenetic techniques, the application of genomic technologies to preimplantation testing offers the promise of cost effective universal methods in preventative medical care. It will be anticipated that SNP microarrays be increasingly used in preimplantation diagnosis of cleavage-stage embryos and blastocysts for both aneuploidies and single-gene disorders.

Preimplantation testing was initiated in the late 1980's to avoid the transmission of severe genetic disorders, but has not yet been widely used due to the high cost of IVF and PGD procedures. Economic and medical considerations, however, favor a universal and affordable access to IVF and PGD services for carrier couples of severe inherited genetic disorders.

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