Review Article

Comprehensive chromosomal screening for preimplantation genetic testing: A mini-review

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Abstract

Preimplantation genetic testing (PGT) consists of a group of genetic tests to evaluate preimplantation embryos before transfer to the uterus during *in vitro* fertilization (IVF). It effectively reduces the incidence of genetic defects at birth by preventing the transmission of inherited diseases to embryos. The use of PGT in IVF clinics has greatly improved clinical pregnancy outcomes for carriers of genetic abnormalities through the selection of embryos that are free from any genetic mutation/chromosomal anomalies. However, the accuracy of PGT in detecting aneuploidies and genetic mutations remains a point of contention due to the varied effectiveness of the techniques used. In recent years, a number of high-throughput assays have been developed to overcome the challenges associated with comprehensive chromosomal analysis. In this review, we will summaries the recent progress in using comprehensive chromosomal screening techniques, including array comparative genomic hybridization, single nucleotide polymorphism array, and next-generation sequencing, to evaluate chromosomal genetic defects.

Keywords: Preimplantation genetic testing, PGT, *In vitro* fertilization, Array comparative genomic hybridization, Single nucleotide polymorphism array, Next-generation sequencing

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INTRODUCTION

With improvements in the understanding of reproduction, infertility treatment has evolved.[1] Infertility due to tubal factor, male factor, and diminished ovarian reserve relies on *in vitro* fertilization (IVF) as a successful treatment option. Preimplantation genetic diagnosis (PGD) is a procedure in infertility clinics that involves genetic testing of biopsy material obtained from oocytes or *in vitro* fertilized embryos and is examined for known molecular anomalies such as chromosomal abnormalities and genetic mutations.[2] On the contrary, preimplantation genetic screening (PGS) refers to screening of patients with advanced maternal age (AMA) or a history of recurrent pregnancy loss for chromosomal aneuploidy. With the help of these procedures, the risk of transmitting genetic disorders is decreased, and complications such as health problems and the psychological and financial burdens associated with termination of a pregnancy may be prevented. The first application of PGD happened in the early 1990s for the detection of X-chromosome-linked diseases by Handyside *et al.* through polymerase chain reaction (PCR)-based sex selection of preimplantation embryos.[3,4] Since then, PGD has been used to diagnose several diseases in different patient groups to

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achieve a healthy pregnancy. Preimplantation genetic testing (PGT) is now the preferred term for PGD. PGT is an approach that effectively reduces the incidences of genetic defects at birth by preventing the transmission of inherited diseases to embryos. The technique has now evolved to incorporate the routine assessment of aneuploidies [PGT for aneuploidy (PGT-A)], chromosome structural rearrangements (PGT for structural rearrangement), and monogenic disorders (PGT for monogenic disorders). Additionally, the use of PGT has been expanded to include diseases that have a polygenic basis (PGT-P).[5]

Advancements in *in vitro* embryo culture have also increased the available options for obtaining genetic material for PGT. Currently, the genetic material for PGT is obtained from a few sources, including polar bodies from oocytes, blastomeres from cleavage-stage embryos, trophectoderm (TE) cells at the blastocyst-stage embryos, and blastocoel fluid in spent embryo culture media referred to as noninvasive PGT.[6,7] The techniques used for comprehensive chromosomal screening have also seen major advances, such as PCR, fluorescent *in situ* hybridization (FISH), chromosomal microarray (CMA), and next-generation sequencing (NGS).[8] The advent of CMA brought about the prospect of finer diagnostic resolution, as it can detect imbalances in the kilobase range, thus demonstrating its superiority over the conventional karyotyping protocol. CMA is a test that includes the detection of gains and losses of segments of DNA or copy number changes, such as loss of heterozygosity, using molecular probes.[9] CMA provides great improvements over the standard chromosomal studies for the detection of chromosomal anomalies that may not be visible through conventional chromosome studies, such as G-banding karyotype. In addition to CMA, newer techniques such as NGS have proven to be more advantageous over microarray-based techniques for PGT. With NGS, the sequenced DNA fragments can be read directly and quantified based on their sequence read numbers. NGS can have various applications in different assays, ranging from whole chromosome aneuploidy detection to medium-size insertions/deletions in chromosomes, along with the detection of monogenic disorders. This article provides an overview of the utility of CMA and NGS in PGT.

Preimplantation Genetic Testing: A Historical Perspective

The first report of successful PGD/PGT was given by Handyside *et al.* in 1990,[4] wherein they identified the Y chromosome at the embryonic stage to rule out the chances of having a child with an X-linked recessive disorder known to exist in the carrier mother. After this first report, the interest of molecular cytogeneticists in the utility of FISH for PGD and PGS began to grow. There were subsequent reports of attempts to use FISH for PGS on either a single blastomere from cleavage-stage embryo or a polar body.[10-12] These procedures were carried out in an attempt to lessen the risk of genetic defects in the child or the occurrence of a spontaneous miscarriage. Subsequently, FISH became the method of choice for the identification of aneuploidy for 12 or more chromosomes.[10,12] However, almost a decade after its clinical introduction for aneuploidy evaluation, the reports from randomized controlled trials (RCTs) suggested poor chances of pregnancies with the use of PGS. Part of the reason for the poor performance of PGS was FISH-based testing, which may not be foolproof against natural biological variations and may involve execution flaws.[13] Due to these limitations, several techniques emerged for comprehensive whole-genome aneuploidy screening. Reports suggest that the error rate of comprehensive whole-genome aneuploidy screening is significantly lower than FISH, and there has been a great improvement in the pregnancy rates.[14]

Chromosomal Microarray Techniques

CMA techniques for identifying submicroscopic imbalances include comparative genomic hybridization (CGH)-based arrays/array CGH (aCGH) and single nucleotide polymorphism (SNP) arrays/SNP array.

Comparative Genomic Hybridization–based Arrays

In CGH-based arrays, DNA from a patient is "compared" to a normal control DNA sample for the identification of areas that are either over- or under-represented in the patient sample.[15] This approach requires fragmentation of chromosomal content from the patient and control, followed by labeling with different fluorescent colors. The dyes are mixed in equal volumes and placed onto the glass, plastic, or silicon surface DNA array. The array consists of multiple probes that are complementary to sequences across the human genome. Complementary binding takes place on the array between DNA and probe in a competitive manner. Using an array reader and digital imaging software, fluorescence intensity is recorded, and the ratio of fluorescence intensities between the patient and control samples is calculated. A ratio of one signifies a normal copy number at the locus or the same number of chromosomes. A ratio of greater than one indicates the hybridization of a greater amount of a patient's DNA at a particular location than the control. This, in turn, suggests

a gain of a chromosome/chromosomal segment (trisomy or duplication/gain). On the contrary, less DNA hybridized on patient DNA to the probe suggests the loss of a chromosome/chromosomal segment (monosomy, deletion, or loss), which would yield a ratio of less than one. Typically, the number of probes in clinical CGH arrays ranges from a few thousands to lakhs, while research CGH arrays usually contain millions of probes. The types of probes and their distribution define the resolution and diagnostic reliability of aCGH.[16]

Single Nucleotide Polymorphism-based Array

SNPs are defined as changes in a single base pair of the genome, present in at least 1% of the population.[17] In an SNP microarray, probes consist of regions of DNA (-20–60 bp) that vary by a single base pair between individuals, and hence, they are called SNPs. On an array, more than 9,06,600 SNPs can be evaluated throughout the genome. This approach requires only a DNA sample of the patient, which is labeled and hybridized to the probes located on the SNP array. The fluorescence probe intensity is measured and compared *in silico* to the normal controls to determine the copy number variations (CNVs). In a clinical setting, hybrid probes are preferably used consisting of both SNP probes and copy number probes. In general, the number of probes on each hybrid array may be as high as 2.7 million. The CNVs of known significance in the range of 50 to 100 kb or higher are reported in clinical laboratories through SNP arrays. In addition to the information on CNVs, the SNP array may be useful for extracting other crucial information about chromosome aneuploidy, polyploidy, chromosomal mosaicism, uniparental disomy, zygosity, etc. However, a major limitation of the SNP array is its inability to detect balanced chromosomal translocations/structural abnormalities.[18]

Comparative Genomic Hybridization Array for Preimplantation Genetic Testing

As mentioned earlier, the initial clinical trials employed FISH as the method of choice for PGT. However, several studies demonstrated that the FISH technique did not increase the delivery rates after embryo biopsy on day 3.[19-22] Hence, in order to mitigate this problem, TE biopsy with comprehensive chromosomal screening was proposed, which had the potential to evaluate all 23 chromosomes for abnormalities.

Conventional CGH was first successfully applied to single cells by Wells *et al.* in 1999.[23] The use of degenerative oligonucleotide-primed PCR was suggested for CGH when starting with a small amount of DNA.[23] Reports of the high frequency of aneuploidy in early human embryos surfaced through two subsequent studies describing the utility of aCGH for analyzing multiple single blastomeres from cleavage-stage embryos.[24,25] These studies were also the first to demonstrate that, apart from the most common anomalies seen in spontaneous abortuses and prenatal samples from early embryos, there were chromosomal defects seen in all of the chromosomes. Furthermore, these studies highlighted the benefits of using aCGH, which could analyze the entire length of the chromosome, unlike FISH, which only targets few loci. It was also the first time that a significant number of partial aneuploidies were reported in human embryos.

Through these early studies, not only was the value of analyzing all chromosomes in early embryos was highlighted, but it was also confirmed that FISH was not a reliable technique for the detection of aneuploidies. It was, however, difficult to apply CGH in clinical practice because of the lengthy protocol, and the wait time to obtain results was longer compared to the standard clinical PGS protocols. Later on, cryopreservation of embryos while waiting to obtain the results was tried by Wilton *et al.*,[26] which consequently led to the birth of the first baby that was born from a fully-karyotyped embryo. This was followed by clinical trials that used aCGH on embryos obtained from patients who presented with recurrent implantation failure, which led to three more births.[27] It was, however, observed that the procedure of freezing the embryos could lead to some loss of viability.

The next approach in the clinical practice was to karyotype the polar bodies rather than the blastomeres, allowing the PGS through aCGH to be completed in 5 days.[28] However, it allowed the detection of maternal meiotic errors, missing out on the ones that arose post-zygotically. Since then, a handful of studies have applied aCGH in clinical practice.

In a study by Yang *et al.*, women undergoing their first IVF cycle with a normal karyotype and no history of miscarriage were divided into two groups: group A ($n = 55$) comprised women who underwent comprehensive chromosomal screening via aCGH on trophectodermal blastocyst sample, and group B ($n = 48$) in which only the morphology of the embryos was assessed. Interestingly, the results showed significantly higher pregnancy rates for group A (69.1%) compared to group B with only 41.7% positive

pregnancy rates ($P = 0.009$).[29] PGT using aCGH has also proven beneficial for women with a history of recurrent miscarriages. In one study, significantly higher implantation rates (52.63 vs 19.15%, $P < 0.001$), clinical pregnancy (69.23 vs 43.91%, $P = 0.0002$), and ongoing pregnancy rates (61.54 vs 32.49%, $P < 0.0001$) were reported after using CGH in 17 women with a history of recurrent miscarriages.[30] Two more studies reported significantly higher implantation rates and lower miscarriage rates with up to 36.3% reduction in women with AMA after employing PGT with aCGH.[31,32]

However, misdiagnosis remains a concern in IVF clinics despite the benefits of aCGH. A technical error rate of 2% per embryo has been reported in a previous study.[33] Another clinical study assessed the error rates per embryo transfer using aCGH and compared it with NGS. An error rate of 1.3% per embryo was slightly higher than the error rate with NGS, which was only 0.7% per embryo. In the case of aCGH, the implantation rate was 63.8%, while it was 69.1% after doing NGS, demonstrating that despite the sensitivity of CMA platforms, errors still occur.[34] Hence, it is imperative to counsel patients on the use of CMA techniques for PGT. Routine prenatal testing should be offered to patients opting for PGT.

Single Nucleotide Polymorphism Array for Preimplantation Genetic Testing

Before its application in PGT, the SNP array was extensively used for genome-wide association studies. A comparative study between FISH and SNP arrays involved the randomization of blastomeres obtained from arrested cleavage-stage embryos in such a way that half of the blastomeres were assessed using SNP arrays, while the other half was evaluated by FISH technique.[35] It was observed that FISH showed 100% mosaicism in the embryos, which was significantly higher than the results obtained through the SNP array which showed only 31% mosaicism despite the fewer number of chromosomes evaluated. These results pointed at the overdiagnosis of mitotic aneuploidy by FISH and the consequent erroneous disposal of euploid embryos. It was later reported that the positive predictive value of TE-based CMA was significantly better than blastomere-based screening.[36] Since then, SNP array has proved to be highly useful in IVF clinics for a comprehensive and sensitive evaluation of chromosomal anomalies.

In a report by Li *et al.*,[37] nearly 3,00,000 genetic markers were identified through SNP microarray which helped in the identification of parental translocation imbalances in embryos easier. Couples with AMA have also benefitted from the PGT SNP array, as higher implantation rates and low miscarriage rates were reported with the use of the SNP array by Schoolcraft *et al.*[38] In a recent study, it has been revealed that the success rate of detecting polygenic and deletional mutations through PGT in early embryos is approximately 98.7% with SNP microarray, whereas the efficiency was 92.5% with NGS. However, NGS proved to be more advantageous for detecting monogenic diseases.[39] A similar study had previously reported the efficiency of SNP in screening monogenic disorders in embryos.[40]

However, inconsistencies in the results of SNP arrays for PGT have been reported when compared to other platforms such as NGS. A recent study reported that of the 105 blastocysts diagnosed with mosaicism using SNP microarray, only about 76.19% mosaicism was reported through NGS for the same embryos.[41]

Keeping in mind these inconsistencies with the use of these high-throughput platforms, there is a growing need for larger and well-designed studies to predict the accuracy of the PGT techniques in detecting chromosomal abnormalities in embryos.

Next-generation Sequencing for Preimplantation Genetic Testing

NGS is a recent advancement in technology that has taken over other diagnostic and analytical techniques. It holds the potential to detect single nucleotide variations, thereby providing more precise genetic information,[42] while also retaining the ability to identify larger chromosomal anomalies such as aneuploidy.[43] So far, NGS is considered to be the most precise and accurate technique for the identification of thousands of data points on a single chromosome.[44] With the automation of the sequencing process in NGS and reduced demand for control samples, the hands-on time and human errors are efficiently decreased.[44-46] As is the case with array technology, a huge number of samples can be run simultaneously on the NGS platform, which cuts down the cost and time without compromising accuracy.[47] In recent years, a number of studies have used NGS for PGT-A, and it has shown staggering compatibility with the use of different biopsy methods.[42,48-51] Results with a high rate of concordance have been achieved with the use of NGS over aCGH.[42] Additionally, NGS has proved to be a better alternative to aCGH as it has shown improvements in pregnancy outcomes when compared to aCGH.[52] As noted previously, NGS has recently proved to be more efficient at detecting monogenic disorders compared to

array platforms such as SNP-based array.[39] A similar study has shown that the mosaicism detection rate by NGS-based PGT was 23.3%, while with SNP-based array, it was only 7.7%. The same study showed improvements in pregnancy rate outcomes through NGS (44.1%) as compared to the SNP-based array (42.38%).[53] Taken together, these studies point to the fact that the use of NGS for PGT in IVF clinics may prove to be more useful than CMA techniques.

FUTURE PERSPECTIVES

NGS is now becoming the method of choice for carrying out PGS and detecting chromosomal aneuploidy.[46,50,54-57] The technique has offered great improvements for PGS for preimplantation embryos compared to array-based comprehensive chromosomal aneuploidy screening methods.[46,58-62] Improvements in the results of mosaicism detection and clinical outcomes of pregnancies have also been seen with the use of NGS as compared to SNP-based arrays.[53] There are several benefits of using NGS over aCGH for chromosomal copy number assessment. These include (1) cost reduction of sequencing owing to high-throughput sequencing technologies, (2) the potential to sequence multiple samples simultaneously in a single experiment, (3) increased chromosomal analysis resolution, which allows increased detection of partial or segmental aneuploidies, (4) decreases the rate of human errors and hands-on-time resulting in consistent results.[50] Larger and well-designed RCTs of PGS using NGS are underway to put an end to the controversies around the use of this high-throughput sequencing.[57] Conclusively, NGS-based PGT represents a reliable and useful alternative to currently available chromosomal analysis techniques to be practiced routinely in IVF clinics.

SUMMARY

PGT has revolutionized the treatment of infertility as it allows the selection of euploid embryos that are unaffected by genetic mutations or chromosomal anomalies that may be carried by parents. Euploid embryos exhibit higher implantation rates, which results in successful pregnancies compared to those carrying mosaicism. Several platforms are now available for comprehensive screening of chromosomal anomalies. FISH was the initial platform used for the screening of the chromosomes. However, due to the inconsistent results obtained in various clinical trials, the technique was quickly replaced with CMAs, such as aCGH and SNP array. These arrays have a higher sensitivity leading to improved pregnancy outcomes in patients with histories of miscarriages or AMA. However, due to the quick advancements in high-throughput technology, RCTs are very limited and there is a growing need for evaluation of clinical efficacy, such as pregnancy and implantation rates for the continuation of routine use of PGT in IVF clinics.

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