Advances in preimplantation genetic testing with next generation sequencing

Abstract

Background: Preimplantation genetic testing (PGT) refers to the determination of an embryo’s chromosomal constituency before implantation and there is a gradual shift in technology towards next generation sequencing (NGS). Numerous biopsy samples such as blastomeres of cleavage stage embryos, trophoderm cells of blastocysts, polar bodies, blastocoele fluid and culture medium can be analyzed with different NGS platforms. NGS technology has been validated and is now clinically applied to detect partial or segmental aneuploidies, chromosomal aberrations including imbalanced translocations, inversions, deletions, duplications, insertions, mosaicism, triploidies and single gene disorders which are often responsible for infertility, spontaneous abortions and fetal malformations or diseases. Comprehensive chromosomal screening for all 24 chromosomes together with single gene disorder detection can be achieved at the same time in a single run using NGS and the analysis of multiple samples with different indications can be done on a single chip. NGS has the ability to detect numerical, structural and genetic abnormalities at a low cost with high efficiency and accuracy.

Keywords: next generation sequencing, preimplantation genetic testing, preimplantation genetic diagnosis, preimplantation genetic screening.

Abbreviations: PGT, preimplantation genetic testing; PGD, preimplantation genetic diagnosis; PGS, preimplantation genetic screening; FISH, fluorescent in situ hybridization; CGH, comparative genomic hybridization; aCGH, array comparative genomic hybridization; NGS, next generation sequencing; WGA, wide genome amplification; CNV, copy number variance; CCS, comprehensive chromosome screening; COGEN, preimplantation and prenatal genetic diagnosis; PGDIS, preimplantation genetic diagnosis international society

Introduction

Preimplantation genetic testing (PGT) is the determination of an embryo’s chromosomal constituency before implantation. Preimplantation genetic diagnosis (PGD) is often used with parents diagnosed with known single gene mutation and chromosome number or structure abnormalities who want to have an unaffected child. Preimplantation genetic screening (PGS) is the screening for aneuploidy especially in patients or couples who have a history of recurrent spontaneous abortions, recurrent implantation failure and advanced maternal age.² The goal of all of the above is to have an unaffected child, avoid the termination of pregnancy or recurrent miscarriages.

Twenty-eight years ago, the first case of a live birth of a normal offspring from PGD for a recessive X chromosome-linked disease was born.² Nowadays; PGT is routinely used to determine single gene disorders, structural and numerical chromosomal abnormalities. The success of PGT has led to over 10,000 babies born worldwide.

Fluorescent in situ hybridization (FISH) was the first diagnostic method used for cytogenetic molecular analysis. However, FISH has several technical limitations including the number of probes required to obtain reliable results and the requirement of specific parent karyotyping prior to testing.³⁻⁵ It analyzes a limited number of chromosomes at a time and thus misses aneuploidies in other chromosomes. These constraints have led to the development of new and improved 24 chromosome copy number analysis technologies including comparative genomic hybridization (CGH), array comparative genomic hybridization (aCGH), digital-PCR, real-time quantitative PCR, SNP microarray and next generation sequencing (NGS), which is the newest method to date. All of these different technologies have their own advantages and disadvantages with respect to the analysis time, cost, resolution, labor needed, procedures and abnormalities which can be detected.¹ This review is a summary of the advances in preimplantation diagnosis with NGS.

Different platforms used in NGS

There are currently three major NGS platforms namely Roche (also known as 454), Illumina and Ion torrent. Life technologies/Ion torrent and Roche/454 life sciences both use emulsion PCR as their amplification method. Illumina on the other hand, uses bridge PCR. As for their sequencing techniques, Roche/454 life sciences use pyrosequencing with a read length of up to 1000bp. However, this method has a high error rate in detecting insertions and deletions within homopolymers. Illumina uses sequencing by synthesis with reversible terminator. The newer platforms of Illumina can yield about 24 million short fragments of DNA sequence per run or “read” which gives it an ultra-high-throughput and makes it cost effective. The main limitation of Illumina is in the increased base substitution error rate. Lastly, we have Life technologies/Ion torrent which uses ion semiconductor sequencing and can read up to 200bp. It has high error rates in specifying insertions and deletions. Although it takes days to have one genome sequence with Illumina, it is the most widely used on the market due to its cost effectiveness, good sequencing throughput and accuracy.⁶ Other emerging sequencing techniques still
need work done in terms of cost, quantity, quality and time.

**Different biopsy sites**

**Blastomere of cleavage stage embryo VS Trophoderm of blastocyst**

After fertilization, each cleavage of the zygote yields two undifferentiated cells called blastomeres. Day 3 cleavage cell blastomeres are usually biopsied to perform analysis for the purpose of PGT. Another, most commonly used stage, at which the biopsy is taken, is the blastocyst stage at day 5 or day 6. Most specifically, cells from the outer layer of the blastocyst are taken, which are known as the trophoderm after gastrulation. Many researchers dispute over which stage of the embryo yields better outcomes. In 2015, Lukaszuk et al.\(^1\) published a prospective case control study in which the day 3 blastomeres were used for aneuploidy analysis by NGS in implantation failure patients. Then, fresh day 5 embryo transfer was performed and the clinical pregnancy rates were compared to a control group in which no ploidy status analysis was done. They concluded that the clinical pregnancy rate was more than 2 times higher in the NGS-blastomere group compared to the control group and the results were statistically significant. Blastomere biopsy has been shown to have several disadvantages. Scott et al.\(^5\) have shown that there is a decreased rate of implantation when the biopsy is performed at the blastomere stage. Mertzanidou et al.\(^9\) revealed that at the cleavage stage, mosaicism is more likely to be present in the embryos. In 2013, Capalbo et al.\(^10\) showed that mosaicism can be avoided or reduced if the biopsy is done on blastocysts instead of blastomeres. In another study by Scott et al.\(^9\) biopsy performed on the blastomere at the cleavage stage had more damaging effects on the embryos. The studies by Jones et al.\(^12\); Capalbo et al.\(^13\) both show that less genetic material is obtained from the biopsy of the embryo at the cleavage stage. This is due to the higher efficiency of trophoderm biopsy. The likelihood for a euploid embryo to reach the blastocyst stage is higher than that of an abnormal embryo. This decreases the number of biopsies that have to be performed in order to obtain a normal euploid embryo from the pool that will be ultimately used for intrauterine transfer. The positive predictive value is therefore increased when trophoderm biopsy is used. A randomized controlled trial by Lee et al.\(^14\) and a meta-analysis by Dahdouh et al.\(^15\) both demonstrated the higher efficiency of trophoderm biopsy.

**Polar bodies**

Treff et al.\(^16\), in 2016, extracted oocytes from mouse models and subjected them to in-vitro maturation after which, they separated the polar bodies from the mature oocytes. Wide Genome Amplification (WGA) followed by NGS was performed on the oocytes, polar bodies and blastocysts to check for aneuploidy. They showed that NGS can be applied to gametes and embryos for the chromosome evaluation as long as the genome is known. Furthermore, Yan et al.\(^17\) performed an analysis on both trophoderm and polar body biopsies using NGS and linkage analysis for a single gene disorder. As the female patient was a carrier of an X-linked chromosome recessive disorder (X-linked hypohidrotic ectodermal dysplasia), successful live birth was achieved. Thus, it was concluded that NGS could be used for analyzing human polar bodies.

**Blastocoele fluid and culture medium**

Recently, non-invasive or minimally-invasive approaches have been developed for chromosomal analysis in PGT. Several studies mentioned the existence of DNA material including chromosomal, genomic and mitochondrial DNA in the blastocoele fluid and culture medium. Xu et al.\(^18\) conducted a study using NGS to analyze fluid from the culture medium of human embryo from day 3 to day 5 and validated their results by comparing it with the comprehensive chromosome sequencing analysis obtained by the corresponding day 5 whole embryo. Their results showed that non-invasive chromosome screening had a specificity of 84% and a sensitivity of 88.2% with positive and negative predictive values of 78.9% and 91.3% respectively. Furthermore, using non-invasive chromosome screening only, they also carried out the first live birth in a patient with a balanced translocation t (14;15) (q22; q24) and repeated the procedure on six more patients which resulted in five healthy chromosomally normal offspring and one ongoing pregnancy. Zhang et al.\(^19\) on the other hand, did single gene PGD using NGS on the DNA found in blastocoele fluid, which is routinely removed from the embryo prior to vitrification to prevent damage by ice crystal formation, and compared the results with the corresponding blastomere embryo. They tested ten genes namely TCIRG1, SCN5A, RHO, EXTL1, SLC4A1, VWF, HSFI4, NPC1, PTC1, and EPS8L3. They obtained a gene level amplification efficiency of 84% which is lower compared to WGA for a single cell which is 90~95%. There is still room for improvements in the chromosomal analysis and gene level analysis for both fluid culture medium analysis and blastocoele fluid analysis. The source of the DNA should be explained and the results from the blastocoele fluid analysis should be validated.

**Frequent abnormalities which can be detected**

**Structural abnormalities: translocation (reciprocal, robertsonian), inversion, deletion, duplication insertion, ring, marker**

Chromosomal aberrations are often responsible for infertility and can be associated with spontaneous abortions and fetal malformations. One of the most common aberrations is the translocation which refers to the breakage and transfer of the broken part to another location on the same chromosome or most frequently on another chromosome. Robertsonian translocation and reciprocal translocation are the two types of translocations that can be identified in the process of PGD. Robertsonian translocation defines the breakage at or near the centromere while reciprocal translocation points out the exchange of materials between non- homologous chromosomes. Inversions, whereby part of the chromosome is reversed end to end, and insertions, where part of one chromosome is deleted from its normal location and inserted into another chromosome, are also often detected during PGD. Up to now, aCGH remains the gold standard to detect those chromosome aberrations. Recently, newer methods have emerged to challenge aCGH more specifically, NGS.

In 2013, Yin et al.\(^20\) showed that NGS can be used to detect chromosomal abnormalities. They analyzed the trophoderm of patients with translocations and inversions using both NGS and SNP array. They also validated their results using qPCR and found that the sensitivity and specificity were 95% and 96% respectively for detecting copy number variance (CNV). They also analyzed for aneuploidies and found that NGS sensitivity and specificity were both 100%. In 2015, Bono et al.\(^21\) conducted a blinded retrospective study to validate the use of a semiconductor NGS for the detection of reciprocal translocations. They used cells from the blastocyst...
stage and embryo cleavage cells from patients previously identified with reciprocal translocations for their analyses and compared the comprehensive chromosome analysis results with those obtained from aCGH. They successfully showed that semiconductor NGS is a robust method of detection for unbalanced reciprocal translocations with a specificity of 99.75% and sensitivity of 100% at the chromosome identification level. As for the identification of chromosomally normal embryo, both their specificity and sensitivity were 100%. The smallest detectable chromosomal segment was 5MB. Other investigators have also further validated NGS for not only reciprocal translocation but also Robertsonian translocations, insertions and inversions. Deleye et al.²² compared Illumina and ion torrent (life technologies) NGS to aCGH by using trophectoderm biopsies at day 5/6 for analyzing numerical and structural chromosome abnormalities. The results from NGS were concordant with those of aCGH. The specificity and sensitivity of NGS were both 100%. They reported the smallest detectable chromosomal abnormality as 4.5MB and also showed that NGS provided an increased signal to noise ratio for lengths up to 3MB. On the other hand, the results are biased at lengths lesser than 3MB due to WGA representation. Following these promising results, in 2016, Zhang et al.²³ was the first to apply CNV-seq NGS for identifying chromosomally normal embryos without the assistance of aCGH for validating the embryos biopsy results of patients who were known to have Robertsonian and reciprocal translocations. These researchers started with cleavage stage biopsy which was ultimately changed to blastocyst biopsy. After having been identified as normal/ balanced euploid, one or two frozen embryos were transferred. They observed an implantation rate of 80%, a 20-week gestation clinical pregnancy rate of 60% and a live birth rate of 70%. They demonstrated that CNV-seq NGS has a high resolution which can detect unbalances near the terminal parts of the p and q arms, and can detect mosaicism down to 20%. Thus, NGS was addressed as a reliable, accurate and clinically efficient method.

The PGD procedure usually refers to differentiating normal/ balanced embryos from chromosomally abnormal/unbalanced aneuploid embryos. However, many normal/balanced embryos may be the carriers of minor chromosomal defects which might be inherited to future generations. Therefore, efforts were made to identify completely normal embryos from carrier embryos. In 2016, Hu et al.²⁴ evaluated 8 patients with reciprocal translocations. In order to obtain the precise location of the breakpoints and modulate breakpoint specific primers, peripheral blood samples from these patients were used for chromosomal microdissection and PCR amplification followed by NGS. Trophectoderm biopsies were taken from the embryos of these patients and PGD was done using NGS. The embryos identified as balanced/euploid embryos were further analyzed using linkage analysis with informative SNP and junction spanning PCR analysis. For patients who did not have completely normal embryos, the carrier embryos were still transferred. Single or double frozen embryo transfer was done in 8 patients resulting in 6 live births (5 carriers with balanced translocation and one with normal karyotype) with karyotypes corresponding to the PGD.

Small deletions and duplications can cause chromosomal syndromes which are associated with copy number variances. With the use of NGS, duplication and deletions as small as 1-2Mb can be detected accurately. Fan et al.²⁵ validated their results using 3 genomic DNA samples with these specific copy number variances and they also used 5 peripheral blood samples from patients with these diseases. They were able to detect a 4.98 Mb 5q35.2-qter deletion associated with Sotos syndrome and a 6.66 Mb 7p22.1-pter deletion associated with 7p terminal deletion syndrome. Moreover, Gui et al.²⁶ were able to specify euploid Charcot-Marie-Tooth disease type A for embryo transfer after NGS-based method using linkage analysis. These results show that NGS can detect very small copy number variances and can help to achieve a disease free pregnancy. Although there are no clinical trials about using NGS for ring and marker structural chromosome abnormalities, NGS seems to have the efficiency and potential to detect them.

Aneuploidy

When the number of chromosomes in a cell deviates from the normal composition, it is called an aneuploidy. It can be due to the addition of a single chromosome (trisomy) or a pair of chromosomes (tetrasomy) or the loss of a single chromosome (monosomy) or a pair of chromosomes (nullisomy). Incorrect number of chromosomes in an embryo often leads to implantation failure, miscarriage and fetal malformations. Fiorentino et al.²⁷,²⁸ designed a 2 phase study for the development, validation and application of NGS for 24 chromosome aneuploidy screening. During the first phase, they evaluated samples with known normal cytogenetic karyotype from cultured amniotic fluid and products of conception and re-evaluated previously tested aCGH blastomeres with their NGS-based protocol and any conflicting results were assessed by QF-PCR. They showed that NGS had 99.98% specificity and a 100% sensitivity to detect aneuploidy according to chromosome copy number assignment. The sensitivity, specificity, positive and negative predictive values were all 100% for NGS in 24-chromosome diagnosis. In the second phase of their study, they analyzed blastocysts prior to embryo transfer using both NGS and aCGH, and conflicting results were re-evaluated by QF-PCR. Their results were similar to the first phase and NGS was found to have a clinical pregnancy rate per embryo transfer of 63.8% and an on-going implantation rate of 62%. Kung et al.²⁹ used NGS to re-analyze previous blastocyst and cleavage stage embryos which were previously tested with aCGH. The sensitivity and specificity were both 100% for NGS and that there was more than 99% concordance between NGS and aCGH. These results were also confirmed by Zheng et al.³⁰ using VeriSeq PGS kit (Illumina) with the smallest detection size of 1.8MB and Sachdeva et al.³¹ using Ion torrent Personal Genome Machine. After the validation of NGS, its clinical applications in PGS cycles were also evaluated. There is a new targeted next generation sequencing (tNGS) platform which has recently undergone preclinical validation for comprehensive chromosome screening by Zimmerman et al.³² They tested the new tNGS platform on both commercially available cell line and found the consistency with conventional karyotyping for the cell lines to be 99.2% and 98.7% for real-time PCR in blastocysts. Yang et al.³³ first re-validated NGS by showing specificity, sensitivity, positive and negative predictive values as 100% by reanalyzing embryos which were previously tested with aCGH. Next, they performed a randomized controlled trial which indicated that NGS and aCGH were statistically similar with respect to clinical pregnancy, ongoing pregnancy and miscarriage rates. Now, NGS is applied for different PGS indications including advanced maternal age, recurrent pregnancy loss and recurrent implantation failure.³⁴

Mosaicism

Mosaicism is defined as having two or more different cells lines with different chromosomal number or structure in one embryo caused by errors in chromosomal segregation during mitosis. When
FISH was used for PGS initially, about 30% of embryos were found to be mosaic. New methods for comprehensive chromosome screening (CCS) such as aCGH, qPCR and SNP array can detect both euploid and aneuploid embryos but are unable to detect mosaicism. However, high-resolution NGS has the ability to detect euploid and aneuploid embryos as well as mosaicism. Maxwell et al. internally validated NGS protocols to detect mosaicism. They compared the results obtained from euploid embryos which were previously tested by both NGS and aCGH but miscarried later. They found that 31.6% were mosaic. Both Maxwell et al. and Munne et al. showed that 9% of all transferred blastocysts leading to ongoing pregnancies were mosaic.

Munne et al. have proposed that there should be a third and intermediary group for mosaic embryos. According to the Controversies in Preconception, Preimplantation and Prenatal Genetic Diagnosis International Society (PGDIS), blastocyst biopsy samples are now categorized into three groups for implantation: euploid embryos (<20% aneuploidy cells), aneuploid embryos (>80% aneuploidy cells) and euploid-aneuploid mosaics (20%-80% aneuploidy). Although the latter often miscarries and implants less frequently, about 40% of mosaic embryos can lead to live birth. Munne et al. reanalyzed the PGS cycles that were previously tested with aCGH by using high-resolution NGS and divided them into mosaic group and euploid group. They found that the euploid group (71%) had a higher implantation rate than the mosaic group (53%), but this difference was insignificant. They also showed that complex mosaic embryos have a significantly lower implantation rate (10%) than mosaic embryos. Therefore, it was proposed that embryos with 20%-40% mosaicism should be selected instead of embryos with >40% mosaicism in accordance with COGEN guidelines. Since complex mosaic embryos had a 10% implantation rate, they proposed that the latter could be used in patients with advanced maternal age and poor reproductive history. PGDIS guidelines suggested that monosomic embryos should be prioritized over trisomic embryos, though Munne et al. presented that trisomic, monosomic or segmental mosaics had similar implantation rates. More research is required to validate the other recommendations made by COGEN and PGDIS regarding which mosaic embryos should be transferred according to the particular chromosomes involved. Caution should be taken when proposing the transfer of mosaic embryos and making an informed decision. For instance, if only one mosaic embryo is found in the first cycle, a second cycle should be performed and if the second cycle is also unsuccessful, the transfer of a mosaic embryo should be considered.

**Triploidy**

Triploidy is a rare chromosomal disorder which affects 2-3% of human pregnancies. It is defined as the presence of three sets of human chromosomes. Triploidy in humans are 69 XXX, 69 XXY, and 69 XYY. According to its etiology, it is usually classified into two groups:

a. Digyny which is when the additional haploid is maternal.

b. Diandry which is when the additional haploid is paternal.

It is associated with multiple abnormalities, partial hydatidiform mole and miscarriages. Maxwell et al. internally validated NGS protocols which were able to detect different triploidy (69 XYY and 69 XXY) that could not be identified by aCGH. Xu et al. found that MALBAC-NGS was unable to detect triploidy while SNP array could. Recently, Marin et al. validated the detection of triploidy in blastocysts using tNGS. They could accurately determine triploidy in their control samples as well as trophectoderm biopsies and found that triploidy during in vitro fertilization is a rare occurrence and is mainly linked to maternal origin.

**Single gene disorder**

Single gene disorder, which is also known as monogenic disease, occurs when a single mutation in a specific gene leads to a hereditary disease. These disorders usually have a late onset at early childhood, impair life quality and sometimes result in a shorter life span. PGD can be used to select embryos which are free from these mutations. In fact, in 1992, the first baby free from cystic fibrosis was born after PGD and since then PGD has been increasingly used to detect monogenic disorders. NGS-based PGD for monogenic diseases was first validated by Treff et al. They showed that the genotype obtained with NGS was 100% consistent with Taqman allelic discrimination test as well as their reference laboratory genotypes for CF: cystic fibrosis and WWS: Walker-Warburg syndrome. Then, Well et al. showed that low-pass whole genome amplification (WGA) and linkage polymorphism before NGS can specify cystic fibrosis mutation. Together with Fragouli et al. it was shown that the whole mitochondrial genome can be analyzed using NGS. Furthermore, it was reported that live births could be achieved by NGS.

Yan et al. described two couples, one with a family history of autosomal dominant disorder (hereditary multiple exostoses) who had trophectoderm biopsies and the other with an X-linked chromosome recessive disorder carrier (X-linked hypohidrotic ectodermal dysplasia) who had trophoderm and polar body biopsies. Both couples underwent PGD using NGS and linkage analysis. The results were confirmed by Sanger sequencing, CGH array and STR analyses. The two couples successfully gave birth to healthy babies. Chen et al. also published an NGS-based SNP haplotype method for HB H disease. They ran the same samples in both Gap-PCR and NGS-based SNP haplotyping, and the latter was able to obtain results for all the samples while Gap-PCR was only able to diagnose one sample. Chen et al. also achieved a live birth.

All of the NGS based methods adopted for monogenic diseases use linkage analysis to obtain their results and require prior analysis of the parents’ lymphocytes and other reference laboratory samples to acquire a locus-specific product. Although this process may require more time and increase the cost of PGD, single gene disorder screening and comprehensive chromosome screening for aneuploidies can be performed on a single chip and for several samples at one time, thereby reducing its cost.

**Embryo transfer**

After NGS analysis which can take a minimum of 24 hours, either fresh embryo or frozen embryo transfer can be performed. Most clinics prefer frozen embryo transfer because NGS is more cost effective when multiple samples are loaded on a single chip and waiting for the appropriate number of samples requires the vitrification of the samples. A randomized controlled trial performed by Coated et al. compared fresh embryo transfer cycles to frozen embryo transfer cycles and found a significant increase in ongoing pregnancies and live birth rate per intended treatment as well as per embryo transfer in the frozen group. Their results also showed that the live birth rate was significantly increased in the frozen embryo transfer group for double embryo transfer. Frozen embryo transfer has the advantage of
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both being cost effective and successful at achieving targeted embryo transfer.

**Limitation and efficiency**

NGS has the ability to detect partial or segmental aneuploidies, chromosomal aberrations including imbalanced translocations, inversions, deletions, duplications, insertions, mosaicism, triploidies, single gene disorders and even mitochondrial disorders and its detection capability is more precise and accurate than aCGH with the smallest detection size of 1.8 Mb. It is the only technology which can accurately detect mosaicism. Comprehensive chromosomal screening for all 24 chromosomes together with single gene disorder detection can be achieved at the same time in a single run using NGS and the analysis of multiple samples with different indications can be done on a single chip which reduces the cost. NGS is highly automated and has a high-throughput which further reduces the cost. The time for analysis has been shortened and can be used in fresh cycles, but frozen cycles are more commonly performed to save the cost.

One of the limitations of NGS is that it cannot detect triplet repeat disorders such as fragile X syndrome. In order to detect single gene disorders, the phenomenon of allele drop out may occur and lead to erroneous results. Therefore, linkage analysis needs to be performed together with NGS to achieve positive results.

**Conclusion**

The PGT has become more common in clinical practice. Due to the gradual shift in technology towards NGS; numerical, structural and genetic abnormalities could be detected by a highly accurate, very efficient and moderately cheap method.

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**Conflicts of interest**

The author declares that they do not have any conflicts of interest.

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