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SPECIAL ARTICLE

Pre-implantation Genetic Testing for Aneuploidy (PGT-A)

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ABSTRACT

Preimplantation genetic diagnosis (PGD) or embryo selection was first performed in 1989 using PCR for gender selection to avoid X-linked recessive disorder. However, there was a misdiagnosis due to allele drop out (ADO). Therefore, fluorescent in situ hybridization (FISH) was recommended for gender selection and detection of chromosome abnormalities and PCR was for monogenic disorders. Since then, a number of advanced modern analysis methods for preimplantation genetic testing (PGT) of chromosome balance were developed. A more sophisticating comparative genomic hybridization microarray (aCGH) was introduced in 2011 providing detailed copy number variation (CNV) of 24 types of chromosomes (22 pairs, X and Y). A single aCGH protocol was used for PGT of aneuploidy (PGT-A) and PGT of segmental rearrangement (PGT-SR) for every chromosome in one go. Next generation sequencing (NGS) replaced aCGH in 2015 due to its better resolution and lower cost. Single nucleotide polymorphism microarray (aSNP) with karyomapping analysis for simultaneous PGT of monogenic disorders (PGT-M) and PGT-A is still more expensive. In this article, various embryo biopsy and chromosome analysis techniques are discussed. The pros and the cons of each techniques are also included.

Keywords: comparative genomic hybridization microarray (aCGH), embryo selection, next generation sequencing (NGS), pre-implantation genetic testing for aneuploidy (PGT-A), pre-implantation genetic screening (PGS).

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Pre-implantation Genetic Diagnosis (PGD) was first introduced by Alan Handyside in 1989[1]. Sex determination was performed on biopsied single cells from in-vitro fertilization (IVF) embryos at six to eight cells stage by deoxyribonucleic acid (DNA) amplification of Y chromosome-specific sequence in order to avoid hereditary X-linked mental retardation condition. This allows unaffected embryos to be identified and chosen to transfer to the uterus. Traditionally, prenatal diagnosis (PND) using chorionic villus sampling (CVS), amniocentesis or fetal blood sampling (FBS) followed by cytogenetic, biochemical or molecular analysis of cells recovered from the fetus can be performed for couples at risk of having babies with severe genetic condition in particular thalassemias and Down's syndrome[2]. However, in case of the fetus is affected,

abortion is offered as an option. Therefore, PGD is an alternative to PND for monogenic disorders and chromosome abnormalities i.e. preimplantation genetic testing for monogenic disorders (PGT-M), preimplantation genetic testing for aneuploidy (PGT-A) and preimplantation genetic testing for segmental rearrangement (PGT-SR). The article focuses on PGT-A and relevant embryo biopsy and modern analysis technology.

Preimplantation genetic testing for aneuploidy (PGT-A)

In IVF treatment, the choice of selecting best quality embryos for transfer depends on their morphology i.e. number of pronuclei, number and regularity of blastomeres and fragmentation. However, some good quality embryos on the morphology criteria failed to implant. Joyce Harper demonstrated that 46% of human embryos developed chromosomal abnormalities during preimplantation stage using 3-color fluorescent in situ hybridization (FISH)[3]. Using a more sophisticated single cell comparative genomic hybridization (CGH) techniques on 12 embryos, Dagan Wells and Joy Delhanty showed that 75% of preimplantation human embryos developed complicated chromosome abnormalities[4]. Possible reason may be because of the abnormal chromosome composition within the embryos. These may explain the low success rates of IVF and natural conception. For this reason, preimplantation genetic for aneuploidy screening (PGS) or preimplantation genetic testing for aneuploidy (PGT-A) was employed to identify chromosomally balanced or euploid embryos for transfer with the hope to improve pregnancy rates of IVF. Embryos with chromosomally balance are chosen for transfer with the hope that they will have more chance of developing into successful pregnancy with the principle of excluding embryos with abnormal chromosomes. Indications for PGT-A are advanced maternal age, repeated miscarriages with normal parental karyotype and repeated implantation failure.

Embryo biopsy techniques

- Polar bodies biopsy

During preconception period, polar bodies can be taken for analysis. They are unused maternal genetic material which will degenerate very soon. Two famous centers were keen to perform polar bodies biopsy are Yury Verlinsky[5] and Santiago Munne[6]. However, both first and second polar bodies are needed for comprehensive results which is labor intensive. Paternal genetic materials are not included in the analysis, therefore, in the recessive condition all oocytes with mutant allele will be discarded while half of them will be heterozygous if fertilized with sperm with normal allele. Dominant disorder inherited from the father cannot be diagnosed by polar bodies biopsy. Moreover, post-zygotic events cannot be revealed by this technique. Therefore, polar bodies biopsy is not popular elsewhere.

- Cleavage stage embryo biopsy

The very first clinical PGD reports employed cleavage stage embryo biopsy at day 3 when there are 6-8 cells[1]. One or two blastomeres are taken for diagnosis. It does not adversely affect the embryonic development[7]. Cleavage stage embryo biopsy had been the most popular technique during 1990-2010. However, only 1 or 2 cells can be obtained for the analysis which can sometimes be technical restriction. The biopsied single cells may be missing and mosaicism are common for FISH analysis leading to problematic diagnostic conclusion. Amplification failure (AF), allele drop out (ADO) and contamination are major obstacles for PCR analysis leading to misdiagnosis[8]. Moreover, most IVF centers require day 4 embryo transfer, therefore, only 24 hours or less is available for analysis. Cleavage stage embryo biopsy was superseded by blastocyst biopsy since early 2010s worldwide because of the improved embryo culture techniques and the need of more biopsied cells for CGH array analysis.

- Blastocyst biopsy

Until recently, with the improved knowledge of embryo culture that allow IVF laboratory to grow human embryos up to day 5 effectively. At blastocyst stage with about 150 cells, 5-10 trophectoderm cells can be taken for the analysis[9]. More biopsied cells help in facilitating the analysis techniques for both monogenic disorders and chromosome balance, including for microarray and

next generation sequencing (NGS) analyses. The chance of AF and ADO markedly reduced[8]. The number of surviving embryo to blastocyst stage is markedly reduced due to natural selection. This reduces workload and cost for the analysis. Since the endometrium at day 5 post-fertilization is not suitable for embryo transfer, all biopsied embryos are stored under liquid nitrogen waiting for transfer in the future. Therefore, there are more time for genetic testing. For this reason, blastocyst biopsy has become the most popular techniques worldwide[10].

- Blastocyst fluid biopsy

Future technique includes blastocyst fluid biopsy. Blastocyst fluid contains DNA from death cells from trophectoderm and inner cell mass (ICM)[11]. With the present advanced analysis techniques, the analysis of blastocyst fluid is possible. However, validation of accuracy is needed before clinical application.

Molecular analysis techniques

- Fluorescent in situ hybridization (FISH)

PCR was used in the very first cases of PGD for sexing[1]. However, misdiagnosis was encountered. This was because of the event called allele drop out (ADO) where one of two alleles in a heterozygous cell fails to amplify and leads to misdiagnosis[12]. This is a unique problem of single cell PCR. Since then FISH was recommended for chromosome abnormalities and sexing[13]. DNA sequences complimentary to particular chromosomes were used as probes for in situ hybridization. Fluorochromes with different colors were tagged in order to identify up to 5 chromosomes at a time. Original applications of PGT-A using FISH were for inherited chromosome abnormalities i.e. Robertsonian and reciprocal translocations. By identifying chromosomally balanced embryos for transfer, PGT-A helps the couples carrying translocations to avoid recurrent miscarriages and get pregnant with a healthy baby.

FISH is a sensitive, accurate and quick method to identify the particular chromosomes. It can be applied to polar bodies, blastomeres and trophectoderm. However, disadvantages of FISH include hybridization efficiency, split signals and overlapping signals. Original FISH was home grown with a few colors. The popular commercial FISH, Aneu Vysis, comprised 5 colors for chromosomes 21, 18, 13, X and Y. FISH was superseded by CGH array in 2011.

- Comparative genomic hybridization microarray (aCGH)

Comparative genomic hybridization (CGH) is a technique using the testing DNA as a probe labeled with green fluorescent dye to co-hybridize with the control DNA labeled with red fluorescent dye to cultured lymphocytes. Areas with orange signal are interpreted as balanced, green as additional and red as deletion. This reveals copy number variation (CNV) information of the 24 types of chromosomes[4]. However, manual CGH was labor intensive and time consuming. CGH became popular when the probes were transferred onto microarray. The hand on laboratory and analysis, even still quite sophisticated, have become more user friendly and reduce hand on time from 72 to 16 hours[14].

At the beginning of aCGH era, most IVF labs were still doing day 3 embryo biopsy. Soon after that the trend of embryo biopsy shifted to day 5 biopsy which provides more cells for the analysis per embryo and fewer embryos for testing. This reduces the cost of analysis. aCGH provides detailed CNV information of all 24 types of chromosomes in one go[15]. Therefore, aCGH replaced FISH in most PGT-A analysis very soon. The most popular aCGH was 24SURE from BlueGnome which was later taken by Illumina. However, main pitfalls of aCGH include the detection of triploidy, mosaicism and balanced translocation. PGT-A using aCGH was employed with the belief that transferring euploid embryos would improve pregnancy outcomes of IVF.

- Single nucleotide polymorphism microarray (aSNP)

Single nucleotide polymorphism (SNP) is the variations of single base pair without causing disease. SNP can be found every 1,000 bp through out human genome. SNP microarray (aSNP) includes probes for genotyping of SNPs throughout human genome. By comparing with control reference DNA, aSNP can provide CNV information, even though not as good as aCGH. However, aCGH gives the advantage of parental origins information of the unbalanced regions. Moreover,

balanced translocation is also possible to identify by aSNP. Employing SNPs information around the particular genes as haplotype blocks and comparing with references from the members of the family, it is possible to perform haplotyping analysis in the embryos, aka karyomapping[16]. Karyomapping using aSNP can be used as a universal linkage analysis protocol for PGT-M and PGT-A at the same time[17, 18]. This can reduce expenses and time for developing new protocol for each new disease. The only drawback of karyomapping at the moment is that its cost is far more expensive that PCR and aCGH or NGS.

- ***Next generation sequencing (NGS)***

It took 13 years and $3-billion for the Human Genome Project to complete human genome sequencing using Sanger sequencing techniques. At present it only takes 16 hours and $1,000 to do whole genome sequencing using next generation sequencing (NGS) or massive parallel sequencing (MPS). For PGT-A, the sequencing results are compared with the reference sequences. This allows CNV analysis in the embryos[19]. NGS is also used for non-invasive prenatal testing (NIPT) analyzing fetal free DNA maternal plasma[20]. Technically, NGS provides a better sensitivity than aCGH for chromosomal mosaicism detection[21]. However, with its more details results, NGS provides more chance of reporting variants of unknown significance (VUS). The cost for NGS-based aneuploidy testing for PGT-A is lower than aCGH. Therefore, NGS replaced aCGH in 2015 and has become the most popular platform for PGT-A.

Preimplantation genetic screening (PGS) for aneuploidy

A meta-analysis was carried out to assess the benefit of PGS[22]. Live birth rate per woman was the primary outcome. Randomized controlled trials comparing IVF/ intracytoplasmic Sperm Injection (ICSI) with PGS versus IVF/ICSI without PGS were included. Nine trials using 5-color, 7-color, 8-color and 9-color FISH were included. No trial using techniques other FISH met the inclusion criteria. In all studies, embryos with best morphology were transferred in the control group and embryos with chromosomally normal were transferred in the intervention group. In the IVF/ICSI with PGS group live birth rate per woman was significantly lower compared to the IVF/ICSI without PGS group in women of advanced maternal age and women with repeated IVF failure (OR=0.59, 95% CI=0.44-0.81 and OR=0.41, 95% CI=0.20-0.88, respectively). Women with a good prognosis exhibited similar trend, although without statistical support (OR=0.50, 95% CI=0.20-1.26). Both cleavage stage biopsy and blastocyst biopsy show similar results. However, other comprehensive chromosome analysis testing methods i.e. aCGH and NGS were not included in this meta-analysis.

This meta-analysis suggests that PGS using multicolor FISH reduces live birth rates in women of advanced maternal age and those with repeated IVF failure. This may be because of the discard of the embryos with abnormal chromosome testing results which leads to a reduced number of available embryos for transfer. There may be no embryo with normal chromosome results for transfer at all in some PGS cycles. Some biopsy blastomeres or trophectoderm cells with abnormal chromosome testing results are from embryos with chromosomal mosaicism, while the rest of the embryos are chromosomally normal and discarded. In addition, some embryos with abnormal chromosomes may undergo trisomic rescue event and turn out to be chromosomally balanced later on. These embryos, if have a chance to transfer, can produce successful normal pregnancy. Therefore, PGS should not be employed routinely.

Conclusion

Since late 2010s PGT-A, PGT-SR and PGS using day 5 blastocyst biopsy and cytogenetic analysis using NGS have become standard worldwide. With the benefit of optimal blastocyst culture technology and culture medium, blastocyst culture provides more biopsied trophectoderm cells for genetic analysis. Blastocyst freezing following the biopsy provides longer time for the analysis allowing possible laboratory cost efficiency management and cost saving. PGT for inherited conditions i.e. monogenic diseases and chromosomal translocations is for

avoiding the transfer of the affected embryos. PGT for aneuploidy screening aims to improve IVF efficiency by reducing time to pregnancy and the chance of implantation failure and recurrent pregnancy loss. However, there is still no prove for the benefit of PGS in increasing pregnancy rate in IVF. Additional technology that may help in evaluating the prognosis of the embryos includes time-lapse imaging, metabolomic study and mitochondrial DNA functions. It seems like karyomapping using aSNP is the best platform for simultaneously analysis of monogenic disorders and chromosome balance at present. However, long term safety of the procedures is still needed to be confirmed.

Potential conflicts of interest

The author declares no conflict of interest.

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