Reprod Med Biol (2016) 15:13–19
DOI 10.1007/s12522-015-0216-6

REVIEW ARTICLE

Preimplantation genetic diagnosis/screening by comprehensive molecular testing

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Received: 26 May 2015 / Accepted: 1 July 2015 / Published online: 14 July 2015

Abstract Although embryo screening by preimplantation genetic diagnosis (PGD) has become the standard technique for the treatment of recurrent pregnancy loss in couples with a balanced gross chromosomal rearrangement, the implantation and pregnancy rates of PGD using conventional fluorescence in situ hybridization (FISH) remain suboptimal. Comprehensive molecular testing, such as array comparative genomic hybridization and next-generation sequencing, can improve these rates, but amplification bias in the whole genome amplification method remains an obstacle to accurate diagnosis. Recent advances in amplification procedures combined with improvements in the microarray platform and analytical method have overcome the amplification bias, and the data accuracy of the comprehensive PGD method has reached the level of clinical laboratory testing. Currently, comprehensive PGD is also applied to recurrent pregnancy loss due to recurrent fetal aneuploidy or infertility with recurrent implantation failure, known as preimplantation genetic screening. However, there are still numerous problems to be solved, including misdiagnosis due to somatic mosaicism, cell cycle-related background noise, and difficulty in diagnosis of polyploidy. The technology for comprehensive PGD also requires further improvement.

Keywords Microarray · Next-generation sequencing · Preimplantation genetic diagnosis · Recurrent pregnancy loss · Translocation

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Introduction

Recurrent pregnancy loss (RPL) is a common clinical condition affecting approximately 5 % of couples trying to conceive [1]. A significant proportion of RPL is associated with chromosomal etiologies. For example, in 3.5 % of couples with RPL, one of the partners is a carrier of a balanced gross chromosomal rearrangement such as translocation or inversion. These particular cases could be treated by preimplantation genetic diagnosis (PGD). PGD involves chromosomal analysis of the fertilized egg using fluorescence in situ hybridization (FISH). Human fertilized eggs undergo cell division about every 24 h, and single blastomere biopsy of the 8-cell stage embryo at day 3 followed by FISH is the conventional approach for PGD. Three-color FISH can theoretically distinguish a cell with balanced chromosomal content from that with an unbalanced chromosome that will result in pregnancy loss.

Nonetheless, the implantation and pregnancy rates of PGD using the conventional FISH method remain suboptimal, partly due to the technical uncertainty of FISH, mostly due to errors caused by overlapping or split signals. Another problem is chromosomal mosaicism among blastomeres, as discussed in detail below. A large series of

studies show a diagnostic error rate of approximately 10 % [2]. To improve accuracy, FISH using two blastomeres was attempted since it was believed that one or two blastomeres could be taken without damaging the biopsied embryos [3]. However, some studies have indicated that 2-cell biopsy is harmful to the embryo [4]. Another idea is blastocyst biopsy at day 5 to take more trophectodermal cells [5]. However, after the embryo has reached the 8-cell stage, the cells start to compact and the cell size becomes smaller than at the blastomere stage, which might adversely affect the accuracy of the diagnosis [6]. Development of a more secure diagnostic method that can overcome the uncertainty of the conventional FISH method is required.

Comprehensive PGD

Another issue that can affect the pregnancy rate in PGD is the effect of chromosomal aneuploidy. At least 40 % of conceptuses are aneuploid, and most aneuploid conceptuses lead to pregnancy loss [7]. Intrinsically, oocytes undergo errors in chromosome segregation much more frequently than sperm or somatic cells. The origin of the extra chromosome in trisomic fetuses or conceptuses is predominantly maternal [8]. This is because the pachytene checkpoint in the prophase of meiosis I that prevents aneuploidy during gametogenesis is less stringent in oogenesis than spermatogenesis [9, 10]. It also seems likely that the loss of spindle assembly checkpoint occurs during oogenesis when the single egg cell becomes very large [11].

In addition, maternal age considerably affects the rate of chromosomal aneuploidy in conceptuses [8, 12]. The magnitude of the effect is extraordinary: among women under the age of 25 years, ~2 % of all pregnancies are trisomic, but, among women over 40 years, this rate increases to 35 %. Age-dependent loss of meiotic cohesion is suggested to be responsible for the age-dependent increase in oocyte aneuploidy [13, 14]. Moreover, translocation affects the segregation error of non-translocated chromosomes via non-homologous synapsis in meiosis, which is called the interchromosomal effect [15]. This proposed mechanism is based on anecdotal observation, but might increase the aneuploidy of the preimplantation embryos, increasing the rate of pregnancy loss. However, the influence of this effect is so low as to be negligible [16].

Thus, in PGD for a couple with a chromosomal translocation carrier, even if the chromosomal content affected by the translocation, balanced or unbalanced, is accurately diagnosed by FISH, the effect of the aneuploidy is too large for the pregnancy rate to be sufficiently improved. To screen more chromosomes, an increased number of probes has been tried [2]. The application of two or even three rounds of FISH could provide information on 24 chromosomes. However, as far as FISH is concerned, the more probes applied, the greater the chances of diagnostic errors.

Microarray is a useful tool for overcoming these difficulties. The microarray, a tool for comprehensive quantitative analysis of genes, was originally developed for genome-wide expression profiling, in particular, for comparative study between two cell populations. Later, the microarray was used for cytogenetics, and it is now an indispensable tool in molecular cytogenetics to detect submicroscopic deletions and duplications. This technique is a product of a revolutionary idea. In a standard FISH, chromosomes of the samples are placed on a glass slide and labeled probes are hybridized on the slide. In the cytogenetic microarray, numerous probes are placed on the slide, and the genomic DNA of the test samples is labeled and hybridized on the slide. In the earlier studies, two types of platforms were used and compared: microarrays equipped with either oligonucleotide probes or bacterial artificial chromosomes (BAC) clones that have an insert of 200 kb long. The sensitivity of the oligonucleotide microarray was subsequently found to be better [17]. Thus, oligonucleotide microarray is currently the standard technique for the molecular diagnosis of patients with mental retardation or multiple congenital anomalies in clinical settings.

In PGD, only one blastomere or ~5 trophectodermal cells can be used for genetic testing. Whole genome amplification (WGA) is required to obtain sufficient genomic DNA for microarray analysis. However, WGA always involves a degree of amplification bias. This bias affects the results of the cytogenetic microarray to varying degrees (Fig. 1). The amplification bias might present as

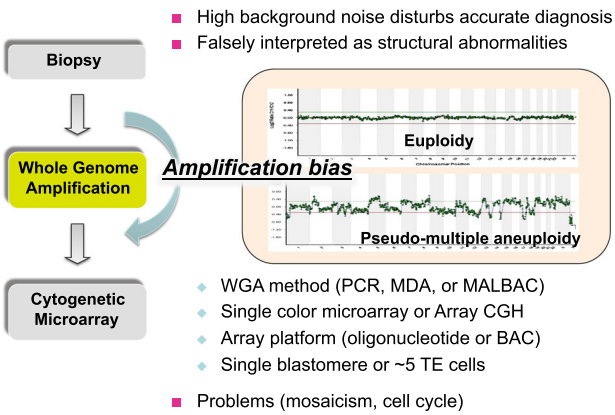

Fig. 1 Problems associated with WGA in cPGD/PGS. Examples for cPGD are shown. *Upper panel* indicates a standard result for euploid sample, whereas lower panel indicates the pseudo-multiple aneuploidy with high background noise produced by the amplification bias or the effect of DNA replication

background noise that is too high to accurately quantify copy numbers or might be falsely interpreted as structural abnormalities [18]. These concerns considerably affect the interpretation of comprehensive PGD (cPGD) results.

Overcoming the amplification bias

Improvements in the cPGD technique have resulted from advances in reducing amplification bias. In earlier studies, cPGD was performed using genomic DNA prepared by a PCR-based amplification method [19]. However, the PCR-based method is always accompanied by an intrinsic amplification bias. Because PCR is based on DNA synthesis, short DNA fragments are preferentially amplified. Another problem is sequence-dependent bias. Amplification of GC-rich regions is difficult because they are more resistant to denaturation and more likely to form secondary structures. Later, a multiple displacement amplification (MDA) method using a phi29 DNA polymerase with strand displacement activity was introduced. Because this polymerase can theoretically resolve secondary structures in template DNA, sequence-dependent WGA bias can be somewhat reduced [20, 21]. However, the bias is still an obstacle for the analysis of a single cell or a small number of cells.

Array comparative genomic hybridization (CGH) is one solution for the amplification bias problem. The sequence-specific amplification bias should be similar among human samples because >99 % of nucleotide sequences are identical. In array CGH, test and reference samples are prepared, labeled with different fluorescent dyes, and simultaneously hybridized to one microarray. When the data are interpreted as a ratio of the two samples, any sequence-specific bias would be offset. Initial CGH analysis was performed on metaphase chromosome specimens, but now on DNA probes spotted or synthesized on the microarray platform [19, 22–24].

Another improvement involves the selection of the microarray probes. The standard oligonucleotide microarray, which shows good performance in clinical pediatrics, is considerably influenced by WGA bias. For WGA-amplified samples, BAC arrays were found to work better because the large size of the probes can dilute the effect of the amplification bias at individual sites [25]. Recently, new WGA technology that can significantly reduce the amplification bias has led to a breakthrough in this field. The method is based on MDA, but the DNA fragments synthesized in the initial amplification cycles form a loop that prevents further amplification, which is called multiple annealing and looping-based amplification cycles (MALBAC) [26, 27]. The combination of this amplification

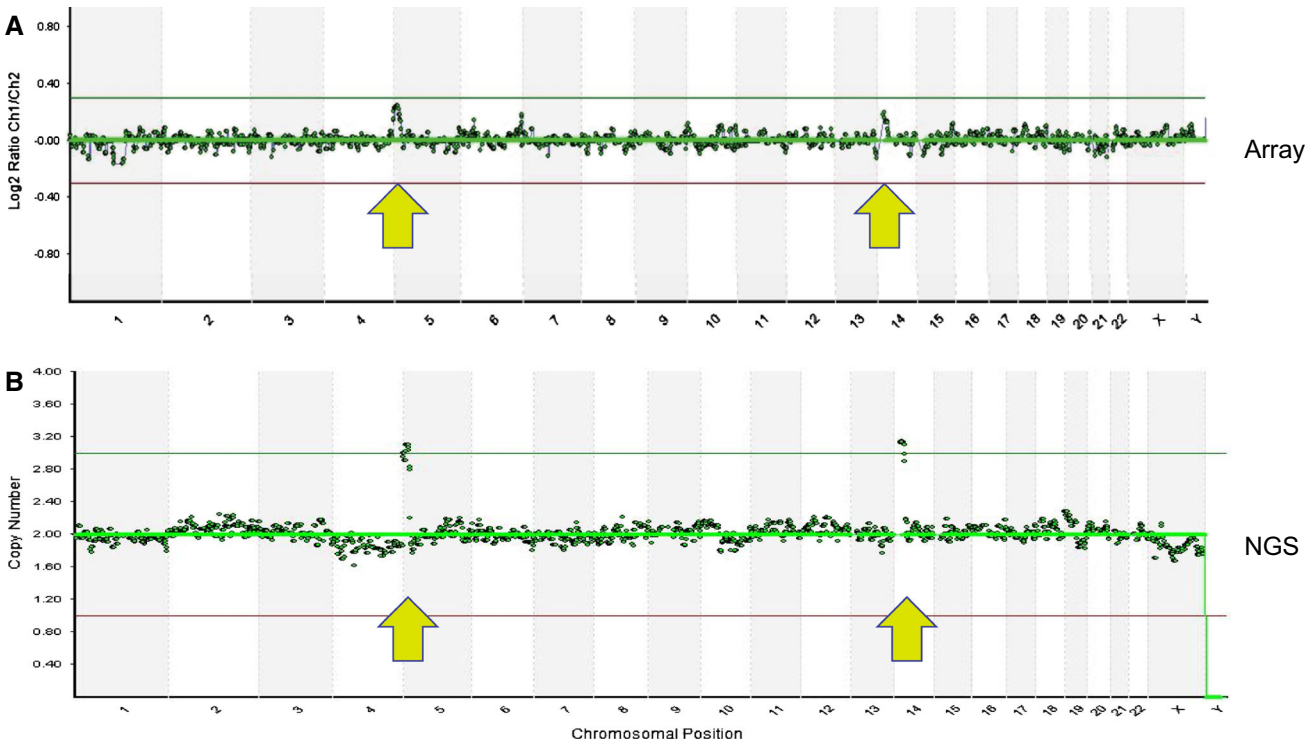

Fig. 2 Comparison of cPGD by BAC array and NGS. Single cell from EBV-transformed lymphoblastoid cell line with 47,XX,+der(14)t(5;14)(p14.3;p13.2) was subjected to WGA followed by analyses with array CGH using BAC array (**a**) or NGS (**b**). This cell line carries 20.9 Mb partial trisomy at 5p and 13.2 Mb partial trisomy at 14p. In this case, sensitivity appears better in NGS than BAC array

method and the array CGH on the BAC array has yielded good performance in PGD [28]. Currently, the BAC array-based protocol (3000 probes per haploid genome) is becoming the standard technique in cPGD for translocation carriers (Fig. 2a).

On the other hand, in the case of the oligonucleotide microarray, dilution of the effect of the amplification bias could be achieved by altering the setup of the window when the raw data are being analyzed. The processed data obtained by averaging the signal intensities of the probes within the window appear to be reasonably accurate. More recently, an array platform specific for PGD was also designed by the selection of probes that are not subject to an amplification bias [29]. These efforts have improved the quality of the cPGD data from the molecular biology research level to that of the clinical laboratory test.

Problems remaining

One of the problems still affecting the interpretation of the results is mosaicism. Blastomeres in cleavage stage embryos show a high rate of mosaicism for aneuploidy, as well as structural abnormalities [30]. In general, chromosome segregation is strictly regulated by the spindle assembly checkpoint. However, in the oocyte or blastomere, where the protein components are diluted in a large cell volume, the function of the spindle assembly checkpoint is transiently deficient, leading to a high rate of mosaicism in this period [11]. This phenomenon raises a fundamental question of whether data obtained from a single blastomere can represent the data of the embryo. Thus, sampling of 3–5 cells by trophectoderm biopsy is now becoming a mainstream approach in cPGD.

Aneuploidy mosaicism in the blastomere stage leads to another aspect that complicates the interpretation of the PGD results: self-correction. A considerable number of embryos showing aneuploidy in the blastomere biopsy undergo self-correction and become euploid embryos during further culture [31, 32]. This means that a low rate of mosaicism might be insignificant. Experimental data using a mixture of DNA from euploid cells and aneuploid cells show that samples with mosaic rates of more than 25 % can be detected by cytogenetic array [33]. This detection rate might be reasonable for missing the low rate of mosaicism of aneuploids intentionally.

Next, cell cycle-related problems also affect the results. In humans, DNA replication starts at more than 10,000 sites throughout the genome. During S phase, the DNA copy number status is like a patchwork. The genomic regions where the DNA replication has already finished have two copies and the remaining regions still have only one copy. This is also a serious problem because these copy number differences might be falsely interpreted as structural abnormalities or might present as high background noise [34, 35]. To avoid this phenomenon, it might be possible to perform sampling just after the cell division under continuous observation using a live imaging system. If the number of cells for the test sample can be increased by trophectoderm biopsy in the blastocyst stage, the effects of different DNA replication timing in each cell might be reduced.

Further, the detection of polyploidism by cytogenetic microarray is generally difficult. Because triploidy is one of the most frequently observed chromosomal aberrations in the aborted fetus, a failure to detect triploidy might affect the birth rate. Using both 46,XX and 46,XY samples as references, the ratio of sex chromosome signals in the test sample relative to references can give some information regarding the polyploidy, but the results are still ambiguous. Microarrays equipped with probes for genotyping of single nucleotide polymorphisms (SNPs) can be used for the detection of polyploidy, but current SNP arrays are not optimized for WGA [36]. Hopefully, SNP array platforms that can show quantitative signals after WGA will be developed.

Preimplantation screening

As mentioned above, a significant proportion of RPL is associated with chromosomal etiologies. Although one of the partners in 3.5 % of couples with RPL is a carrier of a balanced gross chromosomal rearrangement, some couples have normal karyotypes but undergo RPL due to recurrent fetal aneuploidy. These couples undergo the loss of multiple pregnancies due to trisomy of different chromosomes, called heterotrisomy [37, 38]. Recent studies indicate that greater than 60–90 % of all first trimester miscarriages may be the result of aneuploidy [39]. However, these RPL couples are likely to carry susceptibility for aneuploidy in gametes [40, 41]. Such couples with RPL can be theoretically treated by the screening of 24 chromosomes in PGD, referred to as preimplantation genetic screening (PGS).

A similar situation also arises due to an advanced maternal age [8]. For women above 40 years of age, the miscarriage rate is as high as 45 % [42]. Because most pregnancy losses in this context are due to chromosomal aneuploidy, it is reasonable to imagine that PGS might benefit these couples. In addition, some couples with recurrent implantation failure might be candidates for PGS because a subset of these failures might be due to recurrent chromosomal aneuploidy in their zygotes [43].

Initial PGS attempts involved screening by multicolor and multicycle FISH, but optimal results were not obtained because the number of examined chromosomes was limited

and the resolution of the signal was low [44]. Next, establishment of 24-chromosome screening was achieved by technical improvements in cytogenetic microarrays. PGS is much easier than cPGD because the patients need only the copy number information of the entire chromosome, which is much larger than the unbalanced region of the reciprocal translocations. The European Society of Human Reproduction and Embryology PGD Consortium has reported four times more oocyte retrievals for PGS than for PGD in couples with cytogenetic abnormalities [45]. In Japan, the Japanese Society of Obstetrics and Gynecology will start a 3-year randomized clinical trial to investigate the effectiveness of PGS for couples with RPL or recurrent implantation failure.

However, these patient groups consist of couples with RPL or infertility with heterogeneous etiologies. It is important to identify the couples who can potentially benefit from PGS prior to the procedure. Clinical research combined with genetic analyses should be used to identify susceptibility genes for chromosomal aneuploidy in gametes. Hopefully, personalized medicine for RPL or recurrent implantation failure will one day be established.

Emergence of next-generation sequencing

Since the emergence of next-generation sequencing (NGS), genetic analysis has undergone a dramatic paradigm shift. NGS is a powerful tool that can allow both qualitative and quantitative analyses to be simultaneously performed. Exome analysis is the best example. Previously, patients with possible Mendelian disease were screened by the Sanger sequence for genetic alterations at the nucleotide resolution, whereas multi-exon deletions/duplications were screened by cytogenetic microarray or a different technology, multiplex ligation-dependent probe amplification. In contrast, exome analysis enables genome-wide mutation screening and, at the same time, quantitative analysis of the exome data provides information on multi-exon deletions/duplications [46]. In this sense, NGS might replace the cytogenetic microarray in the near future.

In these days, NGS is commonly used for chromosomal copy number analysis, particularly in the non-invasive prenatal test for trisomy detection. Fetal DNA can be found in maternal plasma as cell-free fetal DNA, but only as a minor fraction ($\sim$10 %). Massive parallel sequencing by NGS followed by quantitative determination according to chromosomal assignment enables the prediction of fetal aneuploidy [47, 48].

In cPGD/PGS, the copy numbers of 24 chromosomes are estimated in a similar way [49]. Indeed, WGA-amplified genomic DNA is sequenced using an NGS-based protocol. Approximately three million sequence reads are mapped and divided into $\sim$1 Mb windows (2500 windows per haploid genome), and then reads in each window are quantified. NGS-based cPGD/PGS appears to be more versatile than microarrays (Fig. 2b). In general, only 0.02 times coverage of the whole genome is enough to identify not only the copy number abnormalities of whole chromosomes, but also those derived from unbalanced translocations. If the sensitivity needs to be increased to detect smaller unbalanced regions, sequence reads can be increased accordingly. The size or position of the window can also be modified for specific cases.

Some ethicists and sociologists have ethical concerns about whole genome embryo sequencing. If people know the nucleotide sequences of all of the genes of their offspring, they would want to know not only the genetic information regarding disease susceptibility, but also that of learning ability or athletic performance. Finally, people might want to change the genetic code of the embryos, leading to "designer babies". For cPGD/PGS, sequence information obtained by the current protocol is equivalent to only 0.02 times coverage of the whole genome. However, it is technically easy to obtain the sequence data of the entire genome. In Japan, the Japanese Society of Obstetrics and Gynecology has not yet permitted PGS in clinical practice. Because the permission of an academic society might lower the hurdle, this issue requires careful handling.

Conclusions

By overcoming the WGA bias, cPGD/PGS has recently become the standard technique in the clinical setting. For couples with a balanced translocation, the sensitivity of the detection of small unbalanced translocations needs to be tested. The procedure needs to be optimized on an individual basis and tailor-made protocols are required. Although it still has some problems, including incidental findings, cPGD/PGS is likely to benefit some couples with RPL or infertility. However, it would be better to determine who can benefit by using a blood test for any susceptibility gene before cPGD/PGS. Of course, it is also important to support the right of refusal when it comes to cPGD/PGS.

Acknowledgments The authors thank Drs. M. Tsutsumi and T. Ohye for helpful discussions. These studies were supported by a grant-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and grants for Research on Intractable Diseases from the Ministry of Health, Labour and Welfare of Japan.

Compliance with ethical standard

Conflict of interest Hiroki Kurahashi, Takema Kato, Jun Miyazaki, Haruki Nishizawa, Eiji Nishio, Hiroshi Furukawa, Hironori

Miyamura, Mayuko Ito, Toshiaki Endo, Yuya Ouchi, Hidehito Inagaki, and Takuma Fujii declare that they have no conflict of interest.

Human rights statements and informed consent Human rights statements and informed consent: All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and its later amendments. Informed consent was obtained from all patients for being included in the study.

Human/animal studies This article does not contain any studies with human or animal subjects performed by any of the authors.

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