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A novel multifunctional haplotyping-based preimplantation genetic testing for different genetic conditions

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STUDY QUESTION: Is there an efficient and cost-effective detection platform for different genetic conditions about embryos?

SUMMARY ANSWER: A multifunctional haplotyping-based preimplantation genetic testing platform was provided for detecting different genetic conditions.

WHAT IS KNOWN ALREADY: Genetic disease and chromosomal rearrangement have been known to significantly impact fertility and development. Therefore, preimplantation genetic testing for aneuploidy (PGT-A), monogenic disorders (PGT-M) and structural rearrangements (PGT-SR), a part of ART, has been presented together to minimize the fetal genetic risk and increase pregnancy rate. For patients or their families who are suffering from chromosome abnormality, monogenic disease, unexplained repeated spontaneous abortion or implantation failure, after accepting genetic counseling, they may be suggested to accept detection from more than one PGT platforms about the embryos to avoid some genetic diseases. However, PGT platforms work through different workflows. The high costliness, lack of material and long-time operation of combined PGT platforms limit their application.

STUDY DESIGN, SIZE, DURATION: All 188 embryonic samples from 43 families were tested with HaploPGT platform, and most of their genetic abnormalities had been determined by different conventional PGT methods beforehand. Among them, there were 12 families only carrying structural rearrangements (115 embryos) in which 9 families accepted implantation and 5 families had normal labor ART outcomes, 7 families only carrying monogenic diseases (26 embryos) and 3 families carrying both structural rearrangements and monogenic diseases (26 embryos). Twelve monopronucleated zygotes (1PN) samples and 9 suspected triploid samples were collected from 21 families.

PARTICIPANTS/MATERIALS, SETTINGS, METHODS: Here, we raised a comprehensive PGT method called HaploPGT, combining reduced representation genome sequencing, read-count analysis, B allele frequency and haplotyping analysis, to simultaneously detect different genetic disorders in one single test.

[†]These authors contributed equally to this study.

MAIN RESULTS AND THE ROLE OF CHANCE: With 80 million reads (80M) genomic data, the proportion of windows (1 million base pairs (Mb)) containing two or more informative single nucleotide polymorphism (SNP) sites was 97.81%, meanwhile the genotyping error rate stabilized at a low level (2.19%). Furthermore, the informative SNPs were equally distributed across the genome, and whole-genomic haplotyping was established. Therefore, 80M was chosen to balance the cost and accuracy in HaploPGT. HaploPGT was able to identify abnormal embryos with triploid, global and partial loss of heterozygosity, and even to distinguish parental origin of copy number variation in mosaic and non-mosaic embryos. Besides, by retrospectively analyzing 188 embryonic samples from 43 families, HaploPGT revealed 100% concordance with the available results obtained from reference methods, including PGT-A, PGT-M, PGT-SR and PGT-HLA.

LIMITATIONS, REASON FOR CAUTION: Despite the numerous benefits HaploPGT could bring, it still required additional family members to deduce the parental haplotype for identifying balanced translocation and monogenic mutation in tested embryos. In terms of PGT-SR, the additional family member could be a reference embryo with unbalanced translocation. For PGT-M, a proband was normally required. In both cases, genomic information from grandparents or parental siblings might help for haplotyping theoretically. Another restriction was that haploid, and diploid resulting from the duplication of a haploid, could not be told apart by HaploPGT, but it was able to recognize partial loss of heterozygosity in the embryonic genome. In addition, it should be noted that the location of rearrangement breakpoints and the situation of mutation sites were complicated, which meant that partial genetic disorders might not be completely detected.

WIDER IMPLICATIONS OF THE FINDINGS: HaploPGT is an efficient and cost-effective detection platform with high clinical value for detecting genetic status. This platform could promote the application of PGT in ART, to increase pregnancy rate and decrease the birth of children with genetic diseases.

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Key words: HaploPGT / read-count analysis / B allele frequency / comprehensive PGT / haplotyping analysis / parental origin of CNV / mosaicism

Introduction

Because of the environmental pollution, increased incidence of endocrine diseases, and advanced reproductive age, the fertility of human beings is getting worse (Zhang *et al.*, 2017; Wang *et al.*, 2020). ART can help to deal with this problem for people who are involved in reproductive issues (Zamani Esteki *et al.*, 2019; Fesahat *et al.*, 2020). In total, 8 010 527 ART treatment cycles were performed from 1997 to 2014 in 39 European countries, and the number of ART treatment cycles was increased from 203 225 in 1997 to 776 556 in 2014 (De Geyter *et al.*, 2018). However, a large proportion of embryos used in ART with good morphology are chromosomally abnormal, which accounts for spontaneous abortion and fetal malformation. Moreover, precise diagnosis of embryos carrying inherited pathogenic mutations is essential to ensure that offspring are without risk of known severely teratogenic or disabling genetic diseases. Therefore, preimplantation genetic testing (PGT) has been presented to minimize the fetal genetic risk and increase pregnancy rate (Fesahat *et al.*, 2020).

The utilization of PGT had been elevated from 2014 to 2017 in the USA (2014: 18 805 cycles; 2017: 54 442 cycles; Roche *et al.*, 2021). PGT has been classified into 3 different types, PGT for aneuploidy (PGT-A), monogenic disorders (PGT-M) and structural rearrangements (PGT-SR). PGT-A refers to the screening of embryos with numerical chromosomal abnormality, which aims to prevent pregnancy loss and IVF failure caused by aneuploidy (Munné *et al.*, 2019, 2020; Chattopadhyay *et al.*, 2021). PGT-M refers to the screening of preimplantation embryos with an inherited single-gene mutation, and only unaffected embryos are transferred to avoid the transmission of monogenic defects (Hu *et al.*, 2021; Vukovic *et al.*, 2021). PGT-SR refers to the screening of preimplantation embryos with chromosome structural abnormality, including balanced and unbalanced chromosomal structural rearrangements. The vast majority of PGT-SR is used for couples with recurrent pregnancy loss to select embryos without pathogenic structural rearrangement for improving pregnancy outcome (Liu *et al.*, 2021). Nowadays, there are also increased demands from families affected by hematological diseases such as thalassemia and hemophilia, thus PGT-HLA can be used to identify embryos, whose HLA compatible with another affected sibling for hematopoietic stem cell transplantation (De Rycke *et al.*, 2020). All these PGT methods can work together to promote the rate of pregnancy, reduce the risk of genetic diseases and even benefit the affected sibling. However, current PGT-A/M/SR are performed by different technical platforms. The high costliness, lack of material and long-time operation of combined PGT methods limit their application.

In order to provide a versatile, efficient and cost-effective solution to achieve all the objectives mentioned above, it is necessary to develop a comprehensive PGT method concurrently testing for PGT-A, PGT-SR, PGT-M and PGT-HLA. The first comprehensive PGT was developed in 2019, called OnePGT (Masset *et al.*, 2019; 2022), which was based on reduced representation genome sequencing (RRGS) and haplarithmisis algorithm to detect the PGT results for 225 embryos from PGT-A/M/SR families. Other authors have also exhibited comprehensive approaches to PGT with next-generation sequencing (NGS) and array, which performed different features (Backenroth

et al., 2019; Chen et al., 2021; Zeevi et al., 2021; Zhang et al., 2021; De Witte et al., 2022). Chen et al. (2021) developed a method based on NGS with 10× depth of parental and 4× depth of embryonic sequencing data to detect PGT results for 53 embryos (Chen et al., 2021). Zeevi et al. (2021) developed Haploseek, based on single nucleotide polymorphism (SNP) microarray, whole-genome sequencing (WGS) and hidden Markov model, to detect PGT results for 27 couples and 151 embryos. Zhang et al. (2021) reported a method, based on FHLA (family haplotype linkage analysis) and cnvPartition algorithm, to detect PGT results for 12 couples and 59 embryos. De Witte et al. (2022) exhibited an analysis platform (Hopla) for PGT-A/M/SR based on RRGS and B allele frequency (BAF) for diagnostic implementation cohort (81 cell lines, 40 families and 128 blastocysts). However, these platforms were not able to perform the different types of genetic analysis completely, such as detection of mosaicism, parental origin of copy number variations (CNVs) and analysis on haploid, polyploid or loss of heterozygosity (LOH).

Here, we developed a new comprehensive PGT method, named HaploPGT (preimplantation genetic testing based on haplotyping; Fig. 1), which combined RRGS, read-count analysis and BAF and haplotyping analysis to reduce sequencing cost and enhance accuracy. Meanwhile, it could provide additional functions, such as identifying embryos carrying balanced translocation, triploid, LOH, as well as parental origin of CNVs in mosaic and non-mosaic embryos.

Materials and methods

Study design

One hundred and eighty-eight embryonic trophoblast samples were collected by blastocyst biopsy on Day 5 or 6 as clinical validation samples. Routinely, only about 3–5 trophoblast cells were taken to minimize the damage to embryos. All samples were provided by Reproductive & Genetic Hospital of China International Trust & Investment Corporation (CITIC)-Xiangya and most of their genetic abnormalities had been determined by different conventional PGT methods beforehand. NGS and comprehensive chromosomal screening was performed to identify segmental and whole chromosomal aneuploidies (Xu et al., 2014). Carriers of structural rearrangement were diagnosed via microdissecting junction region (MicroSeq) with Junction-spanning PCR and sequencing (PCR-seq; Hu et al., 2016). Rearrangement breakpoints and adjacent SNPs were characterized by NGS following MicroSeq. PCR-seq was further used to identify the precise breakpoints. Informative SNPs were chosen for linkage analyses to identify the carrier embryos. The inheritance of monogenic diseases and HLA in embryos were identified by haplotyping method, which were analyzed to contrast with those of reference PGT-M method (Masset et al., 2019). This study was approved by the ethical committee of the CITIC-Xiangya Reproductive & Genetic Hospital (LL-SC-2021-007).

DNA extraction, whole-genome amplification and NGS

Parental genomic DNA (gDNA) was extracted from peripheral blood samples using a Blood/Tissue genomic DNA extraction kit (QIAGEN-69504) according to the manufacturer's instruction. Embryonic trophoblast samples were processed by REPLI-g Single-Cell Kit (QIAGEN-150345) for multiple displacement amplification (MDA)-based whole-genome amplification (WGA) according to the manufacturer's instructions. Then, the genomic DNA was double-digested with two restriction enzymes and ligated to adaptors (the sequences of adaptors are given in Supplementary Table SI). The combination of the two endonucleases was used to cut the genomic DNA to generate 2000–5000 endonuclease sites per 1-Mb region on human genome. Which was different from random method, the fragments generated by endonuclease combination were located on fixed areas, and accounted for 10–20% of the whole genome. In addition, the use of modified adaptors including viscous ends of CATG and GATC were compatible with multiple NGS platforms. The ligated products were then selected by AMPureXP magnetic beads to obtain fragments with specific sequence characteristic and desired size (200–400 bp). Finally, the libraries were amplified by PCR (the sequences of PCR primers are given in Supplementary Table SI) and underwent NGS on an MGI-200 platform (MGI Tech Co., Ltd, ShenZhen, China) on which the paired-end sequencing was used for sequencing and the read length was 100 bp. The output data of each sample should be $\geq$ 20 million reads (M), and quality score of 30 (Q30) >80%. The sequencing data were then used to identify SNPs and indels through being aligned to human reference genome.

NGS data analysis

The sequencing data were aligned to human reference genome (National Center for Biotechnology Information hg19 version) via BWA (Burrows-Wheeler-Alignment Tool, http://bio-bwa.sourceforge.net/). GATK (Genome Analysis Tool Kit, http://www.broadinstitute.org/gatk/) was used to identify SNPs and indels in the genome.

Detection of triploid and LOH

Triploid, diploid and global LOH could be detected through the combination of BAF analysis across the whole genome and Z-test. Partial LOH could be detected by comparing the proportion of heterozygous SNPs in sample genome with that in the normal diploid genome via Circular Binary Segmentation (CBS) algorithm and Z-test. It was worth noting that the Z-test used here was different from that in CNV origin analysis.

CNV origin analysis

Informative SNP sites with specific pattern were selected for CNV origin analysis, where paternal alleles were homozygous A (AA) and maternal ones were homozygous B (BB), denoted as AABB; or paternal alleles were homozygous B (BB) and maternal ones were homozygous A (AA), denoted as BBAA. Thus, the different types of CNV could be identified by the BAF and genotype frequency (AA, BB and AB) of the embryo. In the embryonic genome, the difference between the BAF value of the CNV region and non-CNV region was examined by Z-test. Theoretically, BAF should be about 0.5 in non-CNV region. Duplication could be distinguished by significantly deviated BAF value of AABB-AB or BBAA-AB (|Z-score|>3). If BAF of AABB-AB increased and that of BBAA-AB decreased, it represented maternal duplication. If BAF of AABB-AB decreased and that of BBAA-AB

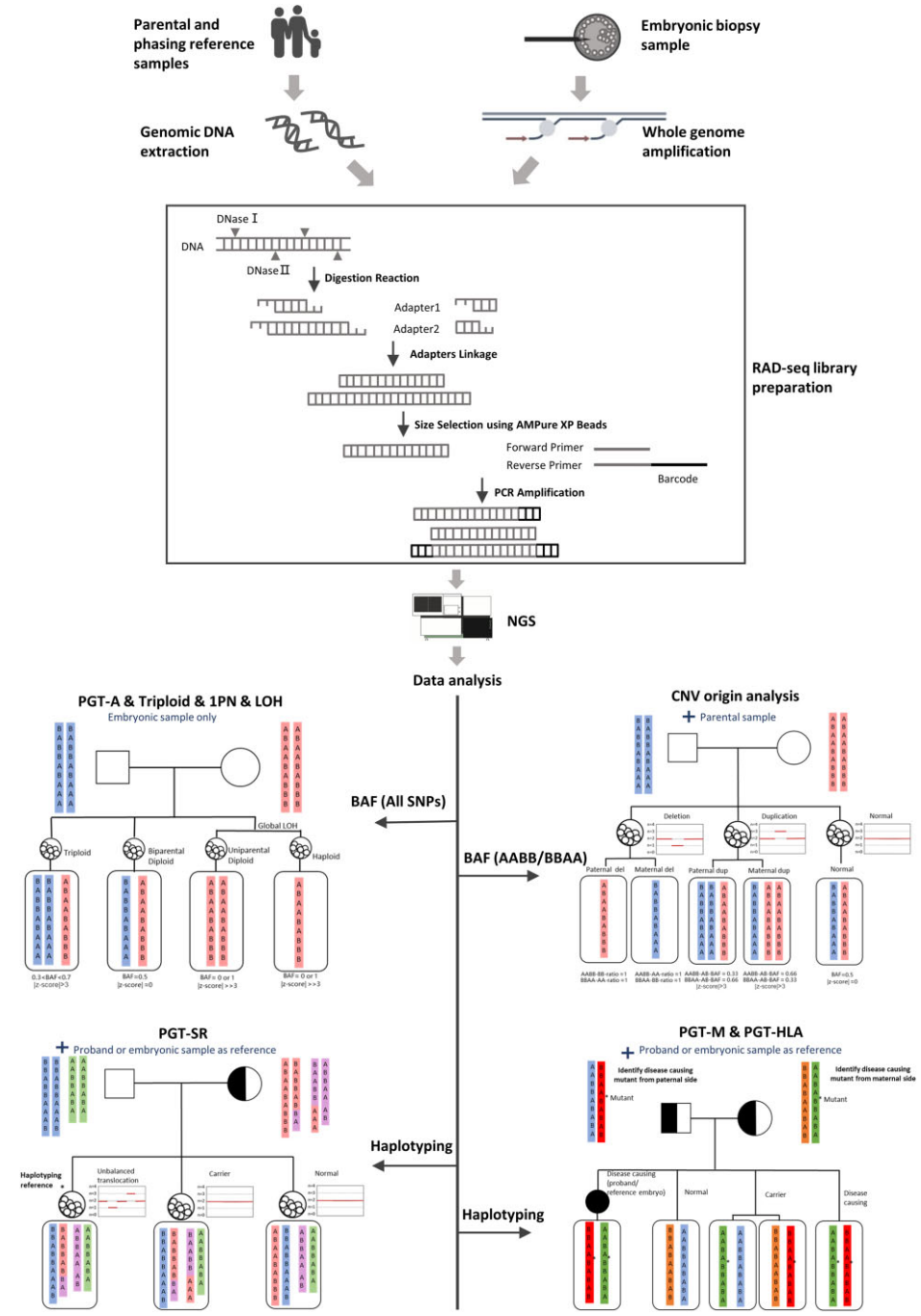


Figure 1. Overview of the HaploPGT. A comprehensive PGT method was developed, and named HaploPGT. HaploPGT recruited copy-number profiling, BAF analysis and haplotyping on RAD-seq data of blastocyst biopsy samples to perform PGT-A, PGT-SR, PGT-M and other extended applications. First, the genomic DNA of parental and phasing samples was extracted, meanwhile embryonic DNA was whole-genome amplified via MDA. Next, RAD-Seq was used to prepare the library and subjected for sequencing. Finally, with the assistance of informative SNPs resulting from the high read depth of RAD-seq, HaploPGT was able to detect triploid, 1PN, LOH and the origin of CNV. The sequencing results were also analyzed based on read-count analysis (PGT-A) and haplotyping (PGT-SR and PGT-M). HaploPGT, preimplantation genetic testing based on haplotyping; BAF, B allele frequency; RAD-Seq, restriction site-associated DNA sequencing; MDA, multiple displacement amplification; LOH, loss of heterozygosity; 1PN, monopronucleated zygotes; CNV, copy number variation; SNPs, single nucleotide polymorphisms; PGT-A, preimplantation genetic testing for aneuploidy; PGT-M, PGT for monogenic disorders; PGT-SR, PGT for structural rearrangements; A/B alleles, the paternal alleles were homozygous A (AA) and maternal ones were homozygous B (BB), denoted as AABB; or paternal alleles were homozygous B (BB) and maternal ones were homozygous A (AA), denoted as BBAA. Thus, the different types of CNV could be identified by the BAF and genotype frequency (AA, BB and AB) of the embryo.

increased, it represented paternal duplication. In the embryonic genome, if the proportion of BB was close to 1 in an embryo whose parental genotype was AABB, it meant the loss of paternal copy, while if that of AA was close to 1, it meant the loss of maternal copy. The patient families with known balanced translocation were used as validation samples for the identification of CNV origin. In these pedigrees, the karyotypes of spouses of the patients with balanced translocation were normal, and the balanced translocation breakpoints of these patients had also been analyzed using other methods. Excluding the embryos with euploidy, aneuploidy and *de novo* translocation, the parental origin of CNV of the remaining unbalanced embryos could be judged from the parent with balanced translocation of the family (Supplementary Table SII).

PGT-A and PGT-SR

PGT-A was analyzed according to a previously described massive parallel sequencing method (Zhang *et al.*, 2013). The number of read counts in each window was normalized via GC correction to eliminate the bias induced by WGA. PGT-SR was dependent on an SNP-based haplotype analysis. For PGT-SR, 2-Mb upstream region and 2-Mb downstream region of two translocation breakpoints were analyzed. CNV breakpoints in the genome were determined by CBS algorithm and Z-test. Mosaicism could also be analyzed based on read counts of CNV. The sequencing data of embryos were normalized and GC corrected in 10-kb windows, then combined to large windows following reference library constructed by the data from 100 pairs of males and females. The normalized value read number 2 (R2) of the large window of embryos was compared with the normalized value read number 1 (R1) of the corresponding window of the reference library. $Log_2RR = log_2 \frac{R2}{R1}$, (RR is ratio of R2 divided by R1). The final logRR value of each region is the average of the log_2RR values of the large windows contained in the region. The copy number (CN) of each chromosome of normal human cells is two, then the CN of each region of the embryo is $CN = 2^{Log_2RR} * 2$, So CN $\geq$ 2.7 was represented for duplication of the region; 2.7>CN $\geq$ 2.3 was represented for duplication of mosaicism of the region; 2.3>CN>1.7 was represented for normal region; 1.7 $\geq$ CN>1.3 was represented for deletion of mosaicism of the region; CN $\leq$ 1.3 was represented for deletion of the region; The ratio of mosaicism was calculated by mosaic = abs(2 − 2*2 log_2RR). Then the analysis of mosaicism could be reported by HaploPGT. A series of samples with different mosaic ratios (10%, 20%, 30% and 50%) was mixed using a cell line sample with known abnormal karyotype and a cell line sample with normal karyotype to validate the accuracy of mosaicism detection, and each condition was repeated three times. The data were analyzed by Chisq test (Supplementary Table SIII).

PGT-M and PGT-HLA matching

PGT-M and PGT for HLA matching (PGT-HLA) were also dependent on an SNP-based haplotype analysis. For PGT-M, 2-Mb upstream region and 2-Mb downstream region of the pathogenic site or the pathogenic gene were analyzed. For PGT-HLA, the 2-Mb upstream region and 2-Mb downstream region of *HLA-A*, *HLA-B*, *HLA-C*, *HLA-DRA* and *HLA-DQB1* were analyzed. The strategy to select informative SNPs referred to previous research (Zhang *et al.*, 2017).

Results

Data from restriction site-associated DNA sequencing (RAD-seq) could support analysis of HaploPGT

For each embryo, the 1PN, triploid, CNV and their origins could be tested with one experiment with HaploPGT, and PGT-A, PGT-SR and PGT-M could also be analyzed. The overview of HaploPGT is shown in Fig. 1. To increase the reliability of haplotyping, sufficient SNPs were required. With the increase of sequencing data size, more informative SNPs could be obtained. However, the proportion of windows containing two or more informative SNPs for haplotyping was almost saturated at 80M (∼97.81%). Meanwhile, the genotyping error rate was stabilized at a low level (∼2.19%; Fig. 2A and B). The low depth area with more than 100-kb at 80M data were shown in Supplementary Table SIV. Therefore, 80M was chosen to balance the cost and accuracy. With this data size, the average quality score of 20 (Q20) was around 96% and Q30 was around 89% in all samples that underwent sequencing, and the median of the number of informative SNPs in 1-Mb window was 25 (Fig. 2C and D). These data revealed that informative SNPs were able to support analysis of HaploPGT in the whole-genome area.

Triploid, haploid and LOH were distinguished by BAF and Z-score

With the low coverage but high read depth of sequencing data, SNPs could be accurately detected across the genome, so that the BAF about all the SNPs was used to identify triploid, diploid and LOH. Twelve 1PN samples and nine suspected triploid samples were collected from different families. In suspected triploid samples, all nine samples were confirmed as triploids resulted from two copies of maternal genome and one copy of paternal genome (Table I and Supplementary Table SV). Triploid embryos had a distinct distribution of BAF mainly around 0.33 and 0.66 across the whole genome due to a unique pattern of heterozygous SNPs (AAB and ABB) and deviated BAF from 0.5 (Z-score >3; Fig. 3A). In 1PN samples, 8 out of 12 samples were biparental diploid, while the rest of them lost heterozygosity globally. 1PN embryos (uniparental disomy, UPD) also had a distinct distribution of BAF mainly around 1 across whole the genome and Z-score ≈ −9. These results were consistent with the reference methods combining SNP array and FISH. This conventional way has confirmed those global LOH samples were in fact 46XX, rather than 23X. However, HaploPGT was not able to distinguish whether they were haploid or diploid resulted from the duplication of a haploid (Supplementary Table SV). In global LOH samples, their BAF of AABB-AB should be mainly distributed around 0 and 1 with significantly less heterozygous SNPs, although the software automatically excluded those SNPs with BAF = 0. Their |Z-score| was very high (>9) across the whole genome (Fig. 3A). Interestingly, a special consanguineous family (case-15) was found in this study and it caused different extent of partial LOH in all their embryos. Via comparing the proportion of heterozygous SNPs in embryonic samples with that in normal diploid controls, the LOH regions larger than 5-Mb were able to be identified (Fig. 3B). Consequently, triploid, 1PN and LOH could be detected through the whole-genomic SNP analysis.

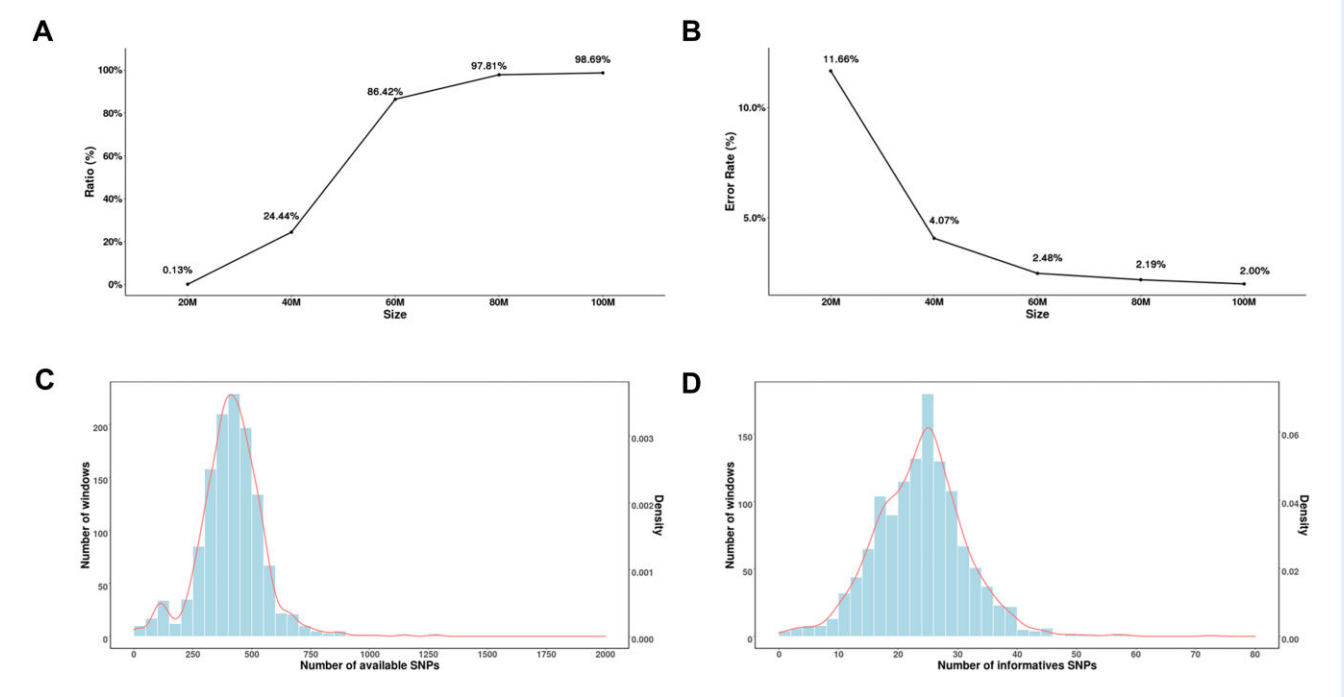


Figure 2. Analysis of sequencing data at different sizes (20M, 40M, 60M, 80M and 100M) and the distribution of SNPs for haplotyping at 80M data. (**A**) The proportion of windows containing two or more informative SNPs for haplotyping at different data sizes. (**B**) The error rate of RAD-seq at different data sizes. (**C**) The distribution of all available SNPs for haplotyping cross all the chromosomes at 80M data. (**D**) The distribution of all informative SNPs for haplotyping cross all the chromosomes at 80M data. SNPs, single nucleotide polymorphisms; RAD-Seq, restriction site-associated DNA sequencing; M, million reads.

Table I Summary of samples used in this study.

Detection	Families	Embryos
PGT-A+SR	12	115
PGT-A + M/HLA	7	26
PGT-A + M+SR	3	26
PGT-A only		
1PN	12	12
Suspected triploid	9	9
Total	**43**	**188**

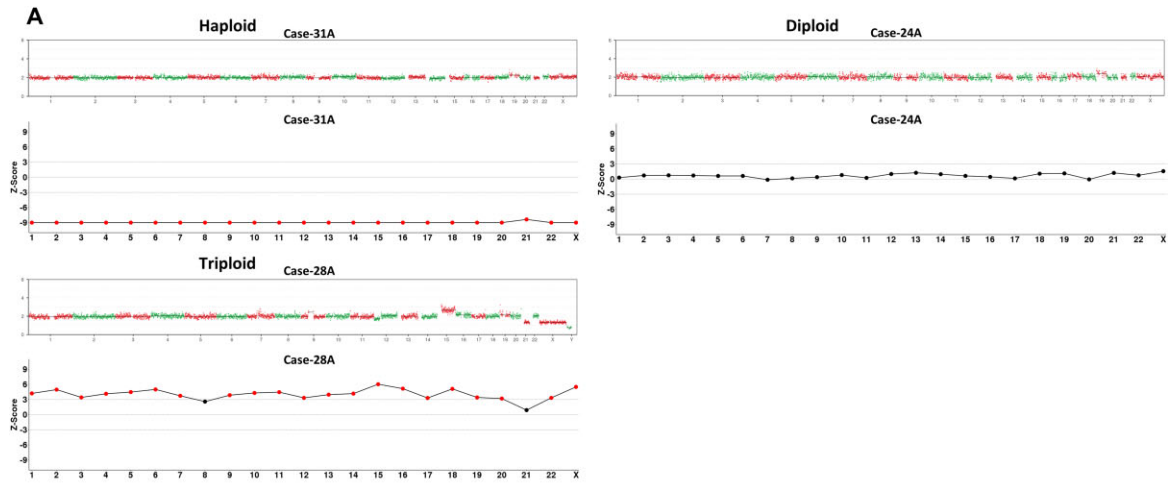
1PN, monopronucleated zygotes; PGT-A, preimplantation genetic testing for aneuploidy; PGT-M, PGT for monogenic disorders; PGT-SR, PGT for structural rearrangements.

Parental origin analysis of CNV in non-mosaic and mosaic embryos

Routinely, segmental or whole chromosome deletion and duplication can be easily observed through PGT-A read-count plot (size >4 Mb). In this study, informative SNPs with a specific pattern (AABB) in parental samples were selected for CNV origin analysis as described in the materials and methods section, which had been validated using samples from family with balanced translocation (Supplementary Table SII). In these pedigrees, there are 54 embryos with unbalanced translocation including 113 CNVs in which parental origin of 109 CNVs was validated consistent with the known origin of CNVs, the other 4 CNVs were inconclusive because of the large fluctuation of z-score (Supplementary Table SII). It showed that the method of BAF and Z-score could accurately determine the origin of embryonic CNVs. Totally, the distribution of available SNPs and informative SNPs at all chromosomes was shown in Fig. 4A and B, which could be sufficient for the CNV origin analysis.

As a result, the origin of deletion could be determined according to the proportion of homozygous genotype (AA or BB) in the embryo, and that of duplication could be detected by assessing the BAF of heterozygous genotype (Fig. 4C and D). Figure 4C shows the CNV origin analysis result of embryo E from case-04 family. PGT-A plots showed only one copy of chromosome X (ChrX) in the female embryo. Since the AABB-BB ratio and BBAA-AA ratio of ChrX were close to 1, it means the remaining chromosome was derived from the maternal side, while the paternal copy was lost in the embryo (Fig. 4C). In another embryo from case-05 family, there were three copies of Chr16 in its genome (Fig. 4D). Since the BAF of AABB-AB on Chr16 was increased and that of BBAA-AB was decreased compared with the expected level of 0.5 (Z-score > 3), we could tell the duplication of Chr16 originated from the maternal side.

Moreover, the origin of mosaic deletion and duplication could also be deducted by combining read count-based PGT-A and SNP genotype-based CNV origin analysis (embryonic mosaicism ≥ 30%; CNV size > 10-Mb). The detection of mosaicism was validated using a series of samples with different mosaic ratios (10%, 20%, 30% and



B

46,XY,t(4;5)(q31;q23)
PLOD3: c.[887C>A];[887=]
POLG: c.[952G>A];[952=]

46,XX
PLOD3: c.[887C>A];[887=]
POLG: c.[952G>A];[952=]

Embryo	Case-15A (reference)	Case-15B	Case-15C	Case-15D	Case-15E	Case-15F	Case-15G	Case-15H	Case-15I	Case-15J
PLOD3	[887C>A]; [887=]	[887C>A]; [887=]	Normal	Normal	[887C>A]; [887=]	[887C>A]; [887=]	[887C>A]; [887=]	**[887C>A]; [887C>A]**	[887C>A]; [887=]	[887C>A]; [887=]
POLG	[952G>A]; [952=]	Normal	**[952G>A]; [952G>A]**	**[952G>A]; [952G>A]**	Normal	[952G>A]; [952=]	[952G>A]; [952=]	[952G>A]; [952=]	Normal	[952G>A]; [952=]
t(4;5) (q31;q23)	Balanced	**Unbalanced**	Normal	**Unbalanced**	**Unbalanced**	**Unbalanced**	Balanced	**Unbalanced**	Normal	**Unbalanced**
LOH(%)	0.73	2.57	2.63	2.44	0.69	1.88	1.65	1.65	3.44	0.67

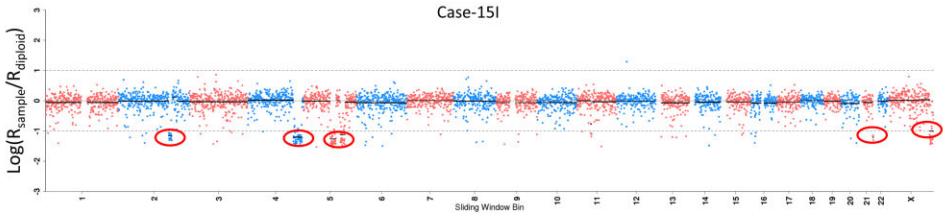
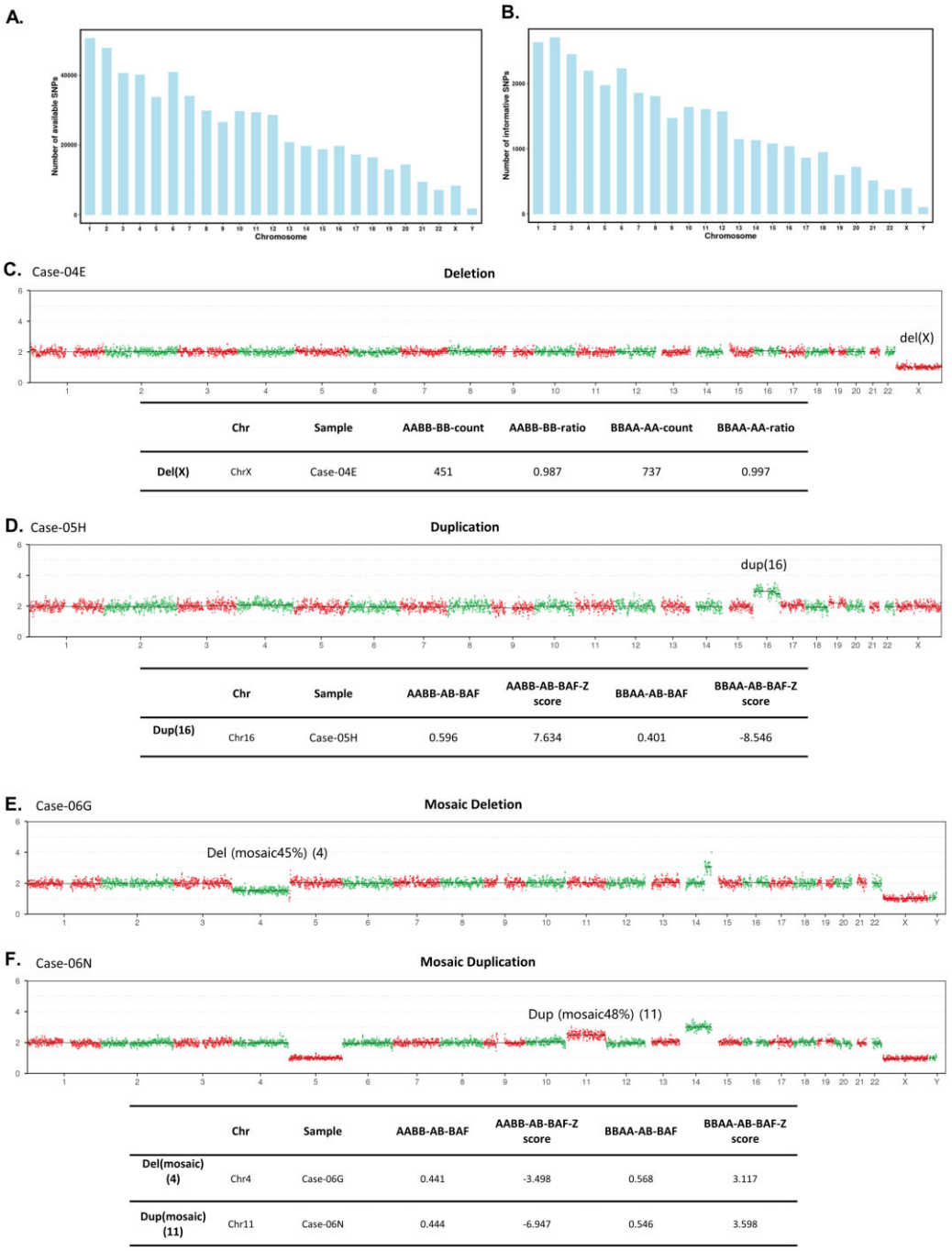


Figure 3. Detection of triploid, biparental diploid, haploid and LOH. (**A**) The situation of identifying triploid, biparental diploid, haploid caused by UPD via calculating the B allele distribution across the whole genome and Z-test is shown. (**B**) Partial LOH occurs in 10 embryos from a consanguineous family (case-15). The father was a carrier of *PLOD3*, *POLG* point mutations and t (4; 5) (q31; q23) balanced translocation. The mother was a carrier of the same *PLOD3* and *POLG* point mutations (*PLOD3*: Represented by a quarter black box or circle; *POLG*: Represented by a quarter gray box or circle). The genotype and LOH percentage of the embryos are listed in the table. Pathogenic variations are marked in bold. The only genotypically normal embryo (case-15I) exhibited 3.44% LOH, which was indicated in the chart below with the LOH regions circled in red. UPD, uniparental disomy; LOH, loss of heterozygosity.

50%). The detection result of a series of samples with different mosaic ratios showed that all mosaicism with a proportion of 10% or more could be effectively detected, which was not significantly different from the theoretical inferred value of percentage of mosaicism in mixed cell lines (Supplementary Table SIII). In embryonic sample case-06G, apart from deletion (Del) (5) (p15.33-p15.32) and duplication (Dup) (14) (q31.1-q32.33), we also detected a mosaic deletion (45%) of Chr4, whose BAF of AABB-AB was much lower than 0.5 and that of BBAA-AB was much higher than 0.5 (Z-score >3) (Fig. 4E). It indicated that the mosaic deletion was due to the lack of maternal Chr4. In another embryonic sample from the same family (case-06N), a mosaic duplication of Chr11 (48%) was reported. Its BAF of AABB-AB was much



	Chr	Sample	AABB-BB-count	AABB-BB-ratio	BBAA-AA-count	BBAA-AA-ratio
Del(X)	ChrX	Case-04E	451	0.987	737	0.997

	Chr	Sample	AABB-AB-BAF	AABB-AB-BAF-Z score	BBAA-AB-BAF	BBAA-AB-BAF-Z score
Dup(16)	Chr16	Case-05H	0.596	7.634	0.401	-8.546

	Chr	Sample	AABB-AB-BAF	AABB-AB-BAF-Z score	BBAA-AB-BAF	BBAA-AB-BAF-Z score
Del(mosaic) (4)	Chr4	Case-06G	0.441	-3.498	0.568	3.117
Dup(mosaic) (11)	Chr11	Case-06N	0.444	-6.947	0.546	3.598

Figure 4. The distribution of SNPs (AABB) for CNV origin analysis (80M) and CNV origin analysis of deletion and duplication. (**A**) The distribution of available SNPs (AABB) at each chromosome. (**B**) The distribution of informative SNPs (AABB) at each chromosome. (**C**) The upper PGT-A plot indicated the deletion of one X chromosome in a female embryo (Case-04E). The table below showed CNV origin analysis on the ChrX deletion. AABB-BB-count represents the number of SNPs with BB genotype on ChrX of the embryo, when father was homozygous AA and mother was homozygous BB at a specific region; AABB-BB-ratio represents the proportion of SNP with BB genotype among all selected sites on ChrX. (**D**) The upper PGT-A plot indicates the duplication of Chr16 in a female embryo (Case-05H). The table below shows CNV origin analysis on Chr16 duplication. AABB-AB-BAF represents the average BAF of SNPs on Chr16 with AB genotype when father was AA and mother was BB; AABB-AB-BAF-Z-score represented the Z-score of the BAF of heterozygous sites in this targeted region from other regions. An absolute value of Z-score greater than 3 indicates the tested value was highly diverged from the mean. (**E**) The upper PGT-A plot indicates mosaic deletion of Chr4 (45%) in a male embryo (Case-06G). The table below shows CNV origin analysis on the Chr4 mosaic deletion. (**F**) The upper PGT-A plot indicates mosaic duplication of Chr11 (48%) in a male embryo (Case-06N). The table below shows CNV origin analysis on Chr11 mosaic duplication. CNV, copy number variation; SNPs, single nucleotide polymorphisms; BAF, B allele frequency; PGT-A, preimplantation genetic testing for aneuploidy; Chr, chromosome.

lower than 0.5 and that of BBAA-AB was much higher than 0.5 (Z-score >3), which meant paternal allele was more than maternal allele, thereby the mosaic duplication was originated from the paternal side (Fig. 4F).

PGT-A and PGT-SR could be detected by haplotyping

All 188 embryonic samples from 43 families were successfully tested for PGT-A, PGT-SR, PGT-M or PGT-HLA (Table I). Among them, there were 12 families only carrying structural rearrangements (115 embryos) in which 9 families accepted implantation and 5 families had normal labor ART outcomes, 7 families only carrying monogenic diseases (26 embryos) and 3 families carrying both structural rearrangements and monogenic diseases (26 embryos). All these familial and embryonic samples were subjected to RAD-seq as described in materials and methods section. The detailed results of the blastocysts were shown in Supplementary Table SVI.

PGT-A and CNV analysis was carried out by the CN profiling in embryonic samples. With this strategy, HaploPGT was able to detect all the whole chromosome aneuploidies and segmental aneuploidies. PGT-SR was performed in 15 families carrying chromosomal structure abnormalities, including 10 reciprocal translocations, 3 Robertsonian translocations, 1 inversion and 1 with combined Robertsonian translocation and inversion (Supplementary Table SVI). However, none of them had a proband reference genome, thus embryos with unbalanced translocation identified in PGT-A could be used as a reference to confirm the breakpoint in each affected family. For instance, in case-01 family, mother carried a balanced reciprocal translocation t (1:7) (q42.3; p15.3). From the PGT-A results of the embryonic genome, 5 out of 9 embryos were shown to have unbalanced translocations (case-01C, case-01E, case-01F, case-01H and case-01I). Therefore, the remaining embryos were investigated via SNP-based haplotype analysis, and the results indicated case-01B, case-01D and case-01G were carriers with balanced translocation, while only one embryo, case-01A, was completely normal (Fig. 5). This normal embryo was then successfully transferred and delivered. The prenatal test result for this embryo was exactly the same as HaploPGT. Other families underwent PGT-SR and their testing results are also presented in Supplementary Table SVI. Among the 141 examined embryos from PGT-SR families, there were 75 embryos from 15 families tested by a reference method, and our HaploPGT results revealed 100% concordance with the reference method, except in case-04 family, where the haplotyping failed due to the lack of an informative reference embryo. In addition, seven of the embryos exhibited unreported mosaicisms (Supplementary Tables SVI and SVII). It is worth noting that HaploPGT could also effectively detect translocation when the breakpoint was very close to the telomere regions (<10-Mb). In all the families with structural rearrangements, 11 karyotypically normal embryos were selected and transferred. Seven of them were successfully delivered and their prenatal test results were consistent with the HaploPGT results.

PGT-A, PGT-M and PGT-HLA could be detected by haplotyping

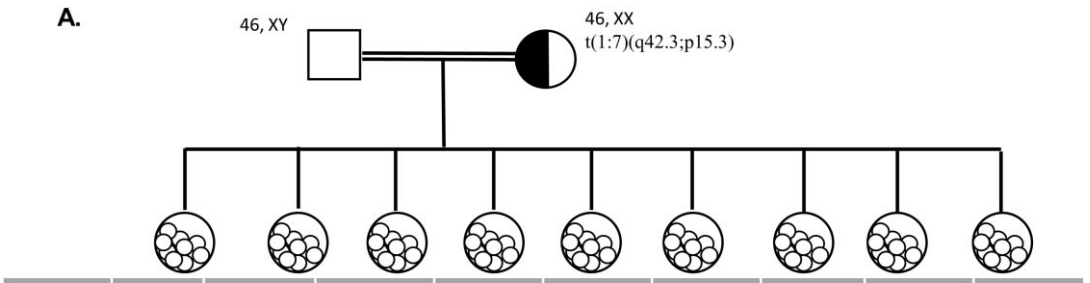
PGT-M was carried out in 52 embryos from 10 families encompassing 14 different monogenic diseases. Six families were affected by single monogenic disease, while the other four families were much more complicated, carrying multiple monogenic disease genes. Their detailed descriptions are listed in Supplementary Table SVII. The inheritance status of pathogenic mutants in the embryos could be confirmed by haplotyping, such as *PLOD3*, *POLG* in case-15 family and *HBB* in case-18 family (Figs 3B and 6). It is worth noting that case-18 family required HLA matching during PGT. The similar haplotype phasing strategy was used to screen for embryos which were HLA compatible with affected a sibling (Fig. 6). In all three families requiring HLA matching during PGT, HLA-matched embryos were successfully identified (Supplementary Table SVII). PGT-M and PGT-HLA results obtained by HaploPGT were consistent with the reference method.

Discussion

For patients or their families who are suffering from chromosome abnormality, monogenetic disease, unexplained repeated spontaneous abortion or implant failure, after accepting genetic counseling, they may be advised to accept testing of embryos from more than one PGT platform, such as PGT-A&PGT-M, PGT-A&PGT-SR, even PGT-A&PGT-M&PGT-SR, to avoid some genetic disease. These PGT platforms work through different workflows, which means high costliness, lack of material, complicated and long-time operation. Through our workflow (i) the amount of data required for WGS was reduced, while the number of the effective SNPs that can be used to construct haplotype was enough; (ii) the haplotype analysis of parents, probands and embryos could be detected by a common workflow; (iii) with a very low amount of sequencing data and a common workflow, the cost, material, complexity and time of operation were greatly reduced for PGT-A, PGT-M and PGT-SR; (iv) our workflow could also exhibit its application on other function, such as the detection of 1PN, triploid, mosaic ratio of mosaic embryos or the parental origin of abnormal CNV. All above embryonic conditions were exhibited in one test, which could give more information to doctors to choose better embryo to transplantation or to conduct scientific research.

Consistent with previous research, a large proportion of 1PN zygotes were actual biparental diploid (Destouni *et al.*, 2018). Therefore, distinguishing diploid embryos from 1PN embryos could increase the number of transferrable embryos in ART clinic. Moreover, triploid and haploid in human embryos have been thought to associate with an abnormal pregnancy called hydatidiform mole (Robinson *et al.*, 2020). HaploPGT not only could detect embryos with triploid but also could distinguish diploid genome with global LOH caused by duplication of a haploid genome. More importantly, LOH also could lead to a rise in imprinting disorders and homozygosity of recessive pathogenic mutations. All these embryos with triploid, haploid, UPD and partial LOH should be excluded for implantation to improve the pregnancy rate and reduce the risk of genetic disease.

Aneuploidy is the most common cause of implantation failure, miscarriage and congenital problems in human populations (Hassold *et al.*, 1996; Grati *et al.*, 2018), so euploid embryos are the preferred choice for implantation in PGT clinic. However, sometimes only mosaic embryos are available for transfer. Evidence showed that several healthy children were born after the transfer of embryos diagnosed with mosaicism in PGT, although the implantation rate and ongoing pregnancy rate of mosaic embryos were much lower than euploid ones (Fragouli



Embryo	Case-1A	Case-1B	Case-1C (reference)	Case-1D	Case-1E	Case-1F	Case-1G	Case-1H	Case-1I
PGT-A	46,XN	46,XN	46,XY,Del(1)(q42.2-q44), Dup(7)(p22.3-p21.1)	46,XN	46,XN,Del(1)(q42.2-q44), Dup(7)(p22.3-p21.1)	46,XN,Del(1)(q42.2-q44), Dup(7)(p22.3-p21.1)	46,XN; Dup(mosaic 35%)(3)(p26.3-p12.2)	46,XN; Dup(1)(q42.2-q44); Del(mosaic 34%)(4)(p16.3-p15.2); Del(7)(p22.3-p21.1)	46,XN; Dup(1)(q42.2-q44); +(mosaic 31%)(2); Del(7)(p22.3-p21.1)
t(1:7)(q42.3;p15.3)	Normal	Balanced	**Unbalanced**	Balanced	**Unbalanced**	**Unbalanced**	Balanced	**Unbalanced**	**Unbalanced**

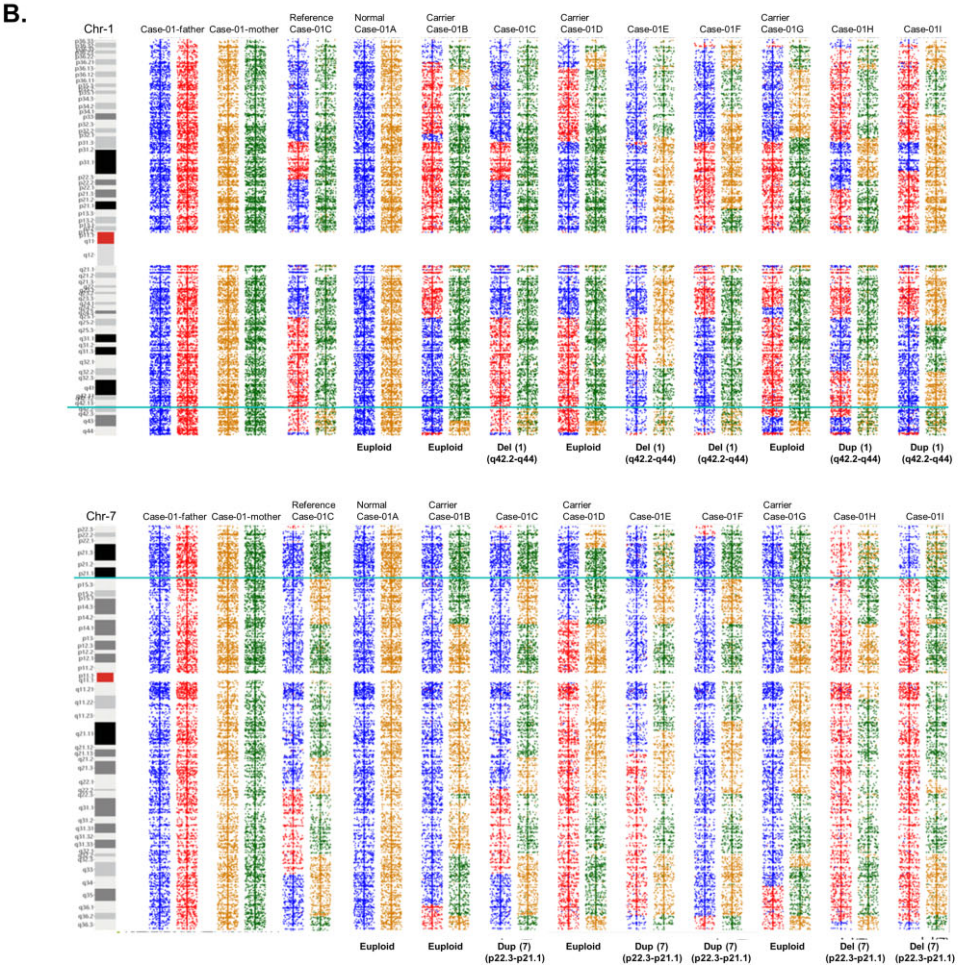
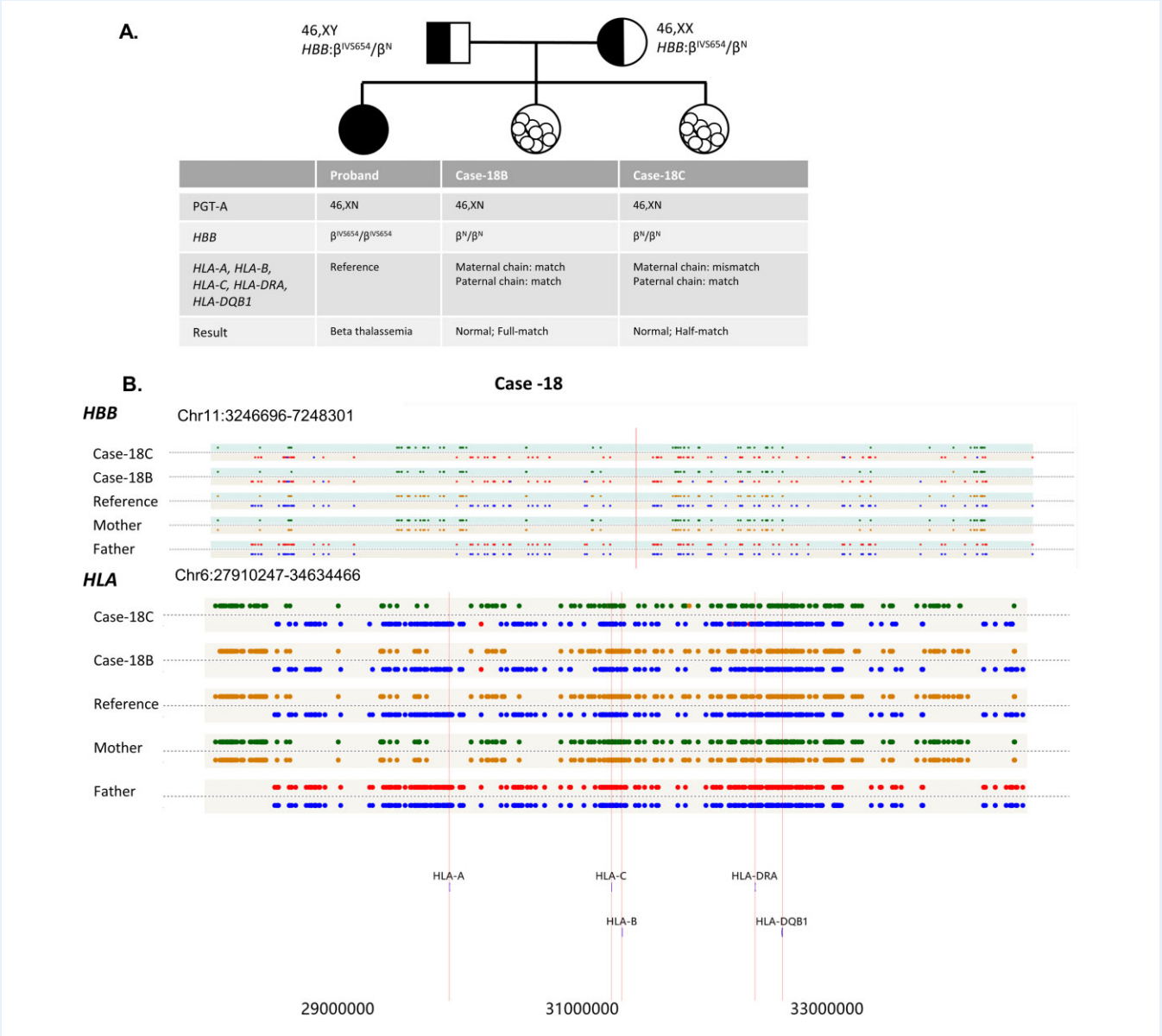


Figure 5. Distinguishing chromosomal rearrangements in embryos via haplotyping. (**A**) Haplotype analysis results of nine embryos from case-01 were shown. The mother was a carrier of t (1:7) (q42.3; p15.3), while father was normal. Among nine embryos, case-01C carrying Del (1) (q42.2-q44) was used as a reference embryo. (**B**) Haplotype analysis of nine embryos from case-01 on Chr1 and Chr7. The blue line indicated the breakpoint of translocation at Chr1q42.3 and Chr7p22.3. Del, deletion; Chr, chromosome.



	Proband	Case-18B	Case-18C
PGT-A	46,XN	46,XN	46,XN
HBB	$\beta^{IVS654}/\beta^{IVS654}$	β^{N}/β^{N}	β^{N}/β^{N}
HLA-A, HLA-B, HLA-C, HLA-DRA, HLA-DQB1	Reference	Maternal chain: match Paternal chain: match	Maternal chain: mismatch Paternal chain: match
Result	Beta thalassemia	Normal; Full-match	Normal; Half-match

Figure 6. Identifying monogenetic disease in embryos via haplotyping and PGT-HLA matching in a family affected by Beta thalassemia (case-18). (**A**) The father and mother were both carriers of β^{IVS654} mutation at *HBB* locus. Their affected daughter was used as a phasing reference to identify the two embryos' inheritance of *HBB* and *HLA* allele from their parents. PGT-M showed both embryos were mutant free, while PGT-HLA reveals only one full matched (case-18B) with the proband. (**B**) Haplotype analysis of two embryos from case-18 on Chr11 and Chr6. The pink line indicated the targeted position in each gene. PGT-M, preimplantation genetic testing for monogenic disorders; Chr, chromosome; PGT-HLA, PGT for human leukocyte antigen matching.

et al., 2017). Therefore, it becomes important to detect the mosaic embryos in clinic to assist the doctors' judgments. Here, HaploPGT could accurately detect the presence of mosaicism. Besides, there were several embryos showing more mosaicism detected by HaploPGT compared with the reference method, which were tested again using HaploPGT, and showed the same result. The reason for the reference method showing less mosaicism might be that some CNVs in special area would not be covered and the sensitivity was lower than HaploPGT. With the assistance of BAF analysis and Z-score, it also possible to identify the parental origin of CNVs in mosaic and non-mosaic embryos, which had been validated using the balanced translocation families (Supplementary Table SII). CNV resulted in microdeletions, microduplications and complex rearrangements of the genome, which disrupt the coding region or alter the gene effect dose. CNV was caused by different mechanisms mainly including inherited CNV and *de novo* CNV. In fetuses where CNV was genetically derived from phenotypically normal parents, the condition tended to be benign but in those whose CNV was *de novo* the condition tended

to be pathogenic (Wu et al., 2017; Shi et al., 2019). *De novo* CNV may be caused by cell division from germ stem cells or postzygotically, and the CNV from paternal line or maternal line may have different contributions to some diseases due to sex-specific development processes of sperms and eggs (Reik and Walter, 2001). Thus, to provide more information for affected families about the repetition risk in following pregnancy and further research the mechanism of CNVs formation, the parent of origin for the CNVs need to be analyzed.

Apart from aneuploidy, chromosomal rearrangements have also been well-known as an important risk factor implicated in miscarriage and infertility. The incidence of balanced translocation in the general population is about 0.29% (Vasilevska et al., 2013). Although most of those carriers with balanced translocation are phenotypically normal, they may suffer from infertility in their adulthood (De et al., 2015). HaploPGT not only could detect reciprocal translocation, Robertsonian translocation and inversion but also rare and more complicated structural abnormalities in combination, to block the transmission to the next generation. With a similar haplotyping approach, PGT-M could eliminate the bias of allele drop-in or drop-out induced by WGA artifacts, which significantly increased the accuracy of mutation detection in embryos (Van der Aa et al., 2013). Moreover, it could facilitate the accurate matching of HLA in families affected by blood diseases.

Comprehensive PGT methods could offer great benefits to all PGT families to conceive babies without genetic abnormalities via a simple, cost-effective all-in-one solution. Till now only five comprehensive PGT methods covering the analysis of PGT-A/M/SR have been developed, OnePGT, Haploseek, low depth WGS-based comprehensive PGT, SNP array-based comprehensive PGT and Hopla. OnePGT mainly used an RRGS method to evaluate aneuploidy and structural rearrangements via a read count-based method and examined monogenic disorders via SNP-based haplotyping (Masset et al., 2019, 2022). Although triploid, haploid and UPD could be deduced from the OnePGT data, OnePGT software did not automatically provide such information. Regarding the Haploseek platform, Zeevi et al. exhibited a cost-effective PGT method covering 0.2×–0.4× human genome through high-throughput sequencing, which was fit for all clinical PGT applications (PGT-A/M/SR), suitable for *de novo* mutations, and could be successfully applied for consanguineous couples, though it needed additional family members. The detection of balanced embryos was performed in the Haploseek platform with sequencing coverage per sample ranging from 0.35 to 0.54, but haplotype analysis of parents and embryos was detected by different workflows (Backenroth et al., 2019; Zeevi et al., 2021). Rather than reducing the sequencing coverage, WGS-based comprehensive PGT utilized another strategy to diminish the sequencing cost by lowering the sequencing depth on embryonic samples to 4× (Chen et al., 2021). It employed a universal read-count analysis for PGT-A, while used haplotype deduction for both PGT-SR, covering balanced translocation, and PGT-M. Another comprehensive PGT based on SNP array has been developed, which also detected aneuploidies, CNV, monogenic mutation and balanced translocation, but does not tell the origin of CNV and mosaicisms. Its PGT-A and CNV analysis are dependent on the SNP allele frequency and Log-R ratio, while PGT-M and PGT-SR utilized linkage analysis. However, SNP array was limited by its fixed number of probes (Zhang et al., 2021). Most recently, De Witte et al. (2022) exhibited a novel analysis platform (Hopla) for PGT-A/M/SR, which could also detect samples for parent-only haplotyping and single-parent haplotyping (autosomal dominant disorders and X-linked disorders). Furthermore, the origin of trisomies in PGT-M embryos was correctly deciphered by Hopla.

Compared with the current comprehensive PGT methods, our study also improved the RRGS, and increased the accuracy of detection of PGT. Through our workflow, the amount of data required for WGS was reduced, while the number of the effective SNP sites was enough to construct haplotype. With 80M size, the proportion of windows containing two or more informative SNPs for haplotyping was 97.81%, the genotyping error rate was 2.19%. These data revealed that informative SNPs were able to support analysis of HaploPGT in the whole-genome area. The haplotype analysis of parents, probands and embryos could be detected by a common workflow. With a very low amount of sequencing data and a common workflow, the cost, material, complexity and time of operation were greatly reduced for PGT-A, PGT-M and PGT-SR. Here, we revealed that HaploPGT yielded 100% concordance with reference PGT-A, PGT-SR and PGT-M methods. Indeed, HaploPGT was also efficient in performing HLA matching, which has been validated in three different families in this study. Besides, our workflow exhibited its application on other function. All above embryonic conditions were exhibited in actual clinical verification in our study, which could give more information to doctors to choose better embryos for transplantation or to conduct scientific research. Therefore, our comprehensive PGT method has a great potential to be applied to the clinical field in the future.

Despite the numerous benefits HaploPGT could bring, it still required additional family members to deduce the parental haplotype for identifying balanced translocation and monogenic mutation in tested embryos. In terms of PGT-SR, the additional family member could be a reference embryo with unbalanced translocation. For PGT-M, a proband was normally required. Another restriction was that haploid and diploid resulting from the duplication of a haploid could not be told apart by HaploPGT, but it was able to recognize partial LOH in the embryonic genome. In addition, it should be noted that the location of rearrangement breakpoints and the situation of mutation sites were complicated, which meant that partial genetic disorders might not be completely detected.

In conclusion, we developed an improved method, named HaploPGT, to provide high accuracy and ability to detect triploidy, LOH, balanced translocation and origin of the CNV. Meanwhile, it comprehensively performed PGT-A, PGT-SR, PGT-M and PGT-HLA matching in human blastocysts, fulfilling all the objectives in PGT. Therefore, it has a great potential to be applied to the clinical field in the future.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

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Authors' roles

L.H., J.D. and B.L. designed and conducted the study, and made the project administration. Y.T., G.L., F.G. and G.L. revised the manuscript. P.X. and X.H. drafted the manuscript and carried out the experiment. D.C., K.K., J.D., D.Z., N.L. and Y.Z. participated in the data collection. L.K., Y.M., J.Z. and B.X. performed data analysis and interpretation. Z.W. and R.D. verified the underlying data. All authors have read and approved the final manuscript.

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Conflict of interest

Haplotyping analysis has been licensed to Basecare Co., Ltd. L.K., Y.M., K.K., D.Z., N.L., J.Z. and R.D. are Basecare Co., Ltd employees. The other authors declare no competing interests.

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