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Validation of a next-generation sequencing–based protocol for 24-chromosome aneuploidy screening of blastocysts

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Objective: To validate a 24-chromosome aneuploidy preimplantation genetic screening protocol based on multiple annealing and looping-based amplification cycle (MALBAC) and next-generation sequencing (NGS).
Design: Single-nucleotide polymorphism (SNP) array and MALBAC-NGS analysis.
Setting: University-affiliated in vitro fertilization (IVF) center.
Patient(s): Fifteen women from whom 30 blastocysts were obtained for genotyping.
Intervention(s): Not applicable.
Main Outcome Measure(s): Chromosomal status comparison of results of array comparative genomic hybridization (aCGH), SNP array, and MALBAC-NGS for 24-chromosome aneuploidy screening.
Result(s): Trophectoderm biopsy samples from blastocysts were first analyzed using array comparative genomic hybridization (aCGH); the embryos with detected with chromosomal abnormalities were rebiopsied, and dissociated into two portions, and subjected to SNP array and MALBAC-NGS for 24-chromosome aneuploidy screening. All 30 samples were successfully genotyped by array CGH, SNP array, and MALBAC-NGS. All blastocysts were correctly identified as aneuploid, and there was a 100% concordance in terms of diagnosis provided between the three methods. In the 720 detected chromosomes, the concordance rate between MALBAC-NGS and array CGH was 99.31% (715 of 720), and the concordance rate between MALBAC-NGS and SNP array was 99.58% (717 of 720). When compared with aCGH, MALBAC-NGS specificity for aneuploidy call was 99.85% (674 of 675; 95% CI, 99.17–99.97) with a sensitivity of 91.11% (41 of 45; 95% CI, 79.27–96.49). When compared with SNP array, MALBAC-NGS specificity for aneuploidy call was 99.85% (676 of 677; 95% CI, 99.17–99.97) with a sensitivity of 95.35% (41 of 43; 95% CI, 85.54–98.72).
Conclusion(s): MALBAC-NGS provides concordant chromosomal results when compared with aCGH and SNP array in blastocysts with chromosomal abnormalities. (Fertil Steril® 2016;■:■–■. ©2016 by American Society for Reproductive Medicine.)
Key Words: Blastocyst, 24-chromosome aneuploidy screening, next generation sequencing (NGS), multiple annealing and looping-based amplification cycles (MALBAC)

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With the development of in vitro fertilization (IVF) technologies, the quantity and quality of the implantable embryos continue to improve. However, the implantation rate of embryos and the IVF success rate remain low, especially for couples of advanced age or with recurrent pregnancy loss or inherited chromosomal rearrangements such as Robertsonian or reciprocal translocations (1).

Chromosome abnormality is one of the major causes for the low embryo utilization rate. Several different technologies have been applied to perform preimplantation genetic diagnosis (PGD) and screening (PGS) on the embryos to improve IVF clinical outcomes (2–4). In the past decade, fluorescence in situ hybridization (FISH) was extensively used for cleavage-stage PGS purposes, but the limited number of tested chromosomes and other technical difficulties resulted in no improvements in the pregnancy and live-birth rates from FISH PGS compared with control groups (5).

With the development of whole genome amplification (WGA) and genomewide genotyping techniques such as array comparative genomic hybridization (aCGH), single-nucleotide polymorphism (SNP) array, and next-generation sequencing (NGS), embryos can be extensively analyzed on all 24 chromosomes (22 pairs of autosomes, and 2 sex chromosomes) for aneuploidy screening (3, 4, 6). Multiple annealing and looping-based amplification cycles (MALBAC) is a newly developed WGA technique (7), and it has been used for genome analysis on single human oocytes (8) and single human sperm cells (9). In addition, MALGAC-NGS has been validated for cleavage-stage PGS for 24-chromosome screening in our center (10).

Blastocyst stage PGS/PGD has been shown to improve detection accuracy (several trophectoderm cells versus one blastomere were collected) as well as biopsy safety compared with cleavage-stage PGS/PGD (11). More and more centers have adopted blastocyst biopsying and have observed improved clinical outcomes compared with cleavage-stage biopsies (12). Therefore, validation of the newly developed MALBAC-NGS method needs to be performed on blastocysts. In our current study, we used blastocysts previously analyzed by aCGH that showed chromosome abnormalities. Trophectoderm biopsies were performed on these embryos; the biopsied samples were separated into two portions, and each was subjected to SNP array and MALBAC-NGS analysis for 24-chromosome aneuploidy screening.

MATERIALS AND METHODS

This study was reviewed and approved by the reproductive study ethics committee at Peking University Third Hospital (research license 2014SZ001). We obtained written informed consent from each participant before the PGD/PGS procedure. Couples with multiple pregnancy loss or inherited chromosomal abnormalities were recruited for this study.

All embryos intended for PGD/PGS purposes were inseminated by intracytoplasmic sperm injection (ICSI) and cultured following standard blastocyst culture procedures. Blastocyst biopsy of three to six trophectoderm cells has been used for aCGH analysis previously. These biopsied blastocysts were frozen using a vitrification method. The embryos with chromosomal abnormalities detected by aCGH were thawed, and they were rebiopsied of 6 to 10 trophectoderm cells. These cells were gently dissected into two portions with similar cell numbers, and they were analyzed by SNP array and MALBAC-NGS, respectively.

We performed aCGH on 24 sure-plus chips (Bluegenome) as described previously elsewhere (10). The SurePlex DNA amplification system (Bluegnome) was used for WGA. The samples and control DNA were labeled with Cy3 and Cy5 fluorophores for approximately 3 hours, with DNA resuspended in dexsulfate hybridization buffer and hybridized overnight. After the samples were washed, a vacuum centrifuge was used to dry the microarray slides, which was followed by laser scanning. BlueFuse Multi software (version 3.1; Illumina) was used to analyze the microarray data on chromosome loss or gain across all 24 chromosomes.

We used a MALBAC WGA protocol to amplify the biopsied trophectoderm cells following the commercial kit protocol from Yikon Genomics (YK001B). MALBAC generates about 2 to 4 μg of DNA required for NGS analysis. With an Illumina HIseq 2500 platform, we sequenced the amplified genome of each sample at approximately 0.04x genome depth. We sequenced a total of approximately 40 million bases, which is equivalent to about 4% of the human genome, obtaining an average genome coverage of 3% for each single cell. Such sequencing throughput yields reproducible copy number variation results with approximately 1 MB resolution to detect the variation (8, 10).

The SNP array was performed on Human CytoSNP-12V2.1 chips (Illumina). The biopsied sample was lysed using an alkaline denaturation buffer (0.2M NaOH) followed by a 4-hour modified multiple displacement amplification protocol using phi29 polymerase to generate template DNA. The DNA product (4 μL, 200 ng) then underwent 13-hour WGA protocol again using phi29 polymerase. Each DNA product then underwent enzymatic end-point fragmentation, and the resuspended DNA samples were dispensed onto Human CytoSNP-12 DNA analysis bead chips (Illumina) and allowed to hybridize for 12 hours. Each CytoSNP-12 bead chip contained ~301,000 SNPs and other genetic markers. Stringent washes were performed to remove unhybridized and nonspecifically bound DNA. The bead chips were dried in a desiccate and scanned using an Illumina iScan Bead Array Reader. Raw data analysis was performed using Illumina Genome Studio software (13).

The chromosomal statuses of the blastocysts using the three methods were compared for validation of the MALBAC-NGS protocol. We also investigated the chromosomal mosaicism phenomenon in these blastocysts.

In addition, we analyzed the concordance of MALBAC-NGS with SNP array and aCGH. To assess the reliability of MALBAC-NGS for aneuploidy detection, the specificity, sensitivity, positive predictive value, and negative predictive value of the test were calculated as Fiorentino et al. described before (6): Specificity: No. of true negatives/(No. of true negatives + No. of false positives); Sensitivity: No. of true positives/(No. of true positives + No. of False Negatives); Positive predictive value: No. of true positives/(No. of true

positives + No. of false positives); and Negative predictive value: No. of true negatives/(No. of false negatives + No. of true negatives).

RESULTS

All 30 blastocysts generated successful testing results for SNP array and MALBAC-NGS. The results are summarized in Table 1. In brief, a total of 30 blastocysts were genotyped using aCGH, SNP array, and MALBAC-NGS. All blastocysts were correctly identified as aneuploid, and there was a 100% concordance in terms of diagnosis provided between the three methods. In the 720 detected chromosomes, the consistency between MALBAC-NGS and aCGH was 99.31% (715 of 720), and the consistency between MALBAC-NGS and SNP array was 99.58% (717 of 720) (Table 1).

Twenty-six blastocysts showed identical results in all three detection methods. Another three blastocysts (numbers 3, 5, and 11) showed that MALBAC-NGS was in agreement with some of the aneuploidies detected by SNP arrays and aCGH but some others were not detected. However, overall, all three methods agreed that the embryos were chromosomally abnormal.

In only one blastocyst (no. 25) was the MALBAC-NGS result not consistent with aCGH or SNP array. In blastocyst no. 25, the deletion of the long arm of chromosome 5(−5q) was not consistent with aCGH or SNP array, but −5q was consistent with the chromosomes involved in the reciprocal translocation (5,21) (q31,q22) from the patient. Moreover, a +21 chromosome was detected instead of a −21q.

A total of 720 chromosomes were assessed. When compared with aCGH, MALBAC-NGS specificity for aneuploidy call was 99.85% (674 of 675; 95% CI, 99.17–99.97) with a sensitivity of 91.11% (41 of 45; 95% CI, 79.27–96.49). The positive predictive value was 97.62%, and the negative predictive value was 99.41% (Table 2). When compared with SNP array, MALBAC-NGS specificity for aneuploidy call was 99.85% (676 of 677; 95% CI, 99.17–99.97), with a sensitivity of 95.35% (41 of 43; 95% CI, 85.54–98.72). The positive predictive value was 97.62%, and the negative predictive value was 99.71% (Table 3).

DISCUSSION

Many studies have reported a high chromosomal abnormality rate in IVF embryos (14, 15), especially in embryos from carriers of inherited chromosomal abnormalities such as

TABLE 1

Summary of results of SNP array, aCGH, and MALBAC-NGS of trophectoderm biopsies from 30 blastocysts.

Blastocyst no.	Indication of PGD/PGS	SNP array	aCGH	MALBAC-NGS
Identical results obtained in all 3 methods (26/30)				
1	46,XX,inv(2)(P11;q13)	+19,XY	+19,XY	+19,XY
2	46,XX,inv(2)(P11;q13)	−22,XX	−22,XX	−22,XX
4	45,XY,rob(13;14)	−14,−16,XX	−14,−16,XX	−14,−16,XX
6	47,XYY	+12,XY	+12,XY	+12,XY
7	46,XX,t(8,20)(q22,p13)	+8p,−20q,XX	+8p,−20q,XX	+8p,−20q,XX
8	46,XX,t(8,20)(q22,p13)	+8q,−20p,XX	+8q,−20p,XX	+8q,−20p,XX
9	46,XX,t(8,20)(q22,p13)	+13,XY	+13,XY	+13,XY
10	PGS	+16,XX	+16,XX	+16,XX
12	PGS	−22,XX	−22,XX	−22,XX
13	46,XY,t(11,13)(q13;q14)	−11q,+13q,XY	−11q,+13q,XY	−11q,+13q,XY
14	46,XY,t(11,13)(q13;q14)	−11q,+13q,XY	−11q,+13q,XY	−11q,+13q,XY
15	46,XY,t(11,13)(q13;q14)	+13, XY	+13, XY	+13, XY
16	46,XX, t(2,10)(q33,q24)	−22,XX	−22,XX	−22,XX
17	45,XX, rob(14,21)	+21,XY	+21,XY	+21,XY
18	45,XX, rob(14,21)	+21,XY	+21,XY	+21,XY
19	45,XX, rob(14,21)	−14,XY	−14,XY	−14,XY
20	46,XY,t(3,5)(q25;q13)	−3q,+5q,XY	−3q,+5q,XY	−3q,+5q,XY
21	46,XY,t(3,5)(q25;q13)	+5,XY	+5,XY	+5,XY
22	46,XY,t(3,5)(q25;q13)	−3q,+5q,XY	−3q,+5q,XY	−3q,+5q,XY
23	46,XY,t(1,3)(p13;q27)	−1p,+3q,XX	−1p,+3q,XX	−1p,+3q,XX
24	46,XX, t(5,21)(q31,q22)	XO	XO	XO
26	46,XX,t(6,12)(p12,p11)	−16,−22,XY	−16,−22,XY	−16,−22,XY
27	46,XX, t(6,12)(p12,p11)	−6p,+12p,XY	−6p,+12p,XY	−6p,+12p,XY
28	47,XYY	−22,XY	−22,XY	−22,XY
29	47,XYY	−16,XY	−16,XY	−16,XY
30	47,XYY	+5,XY	+5,XY	+5,XY
Inconsistency shown in any 2 of the 3 methods (4/30)				
3	46,XY,t(2;5)(q32;q52)	−2q,+5q,XX	−2q,+5q,XX	−2q,XX
5	45,XY,rob(13;14)	+11,−13,XX	+11,−13,XX	−13,XX
11	PGS	−9q,−18,XY	+2p,+2q,+9q,−18,XY[a]	−9q,−18,XY
25	46,XX, t(5,21)(q31,q22)	−21q,XY	−8p,−21q,XY	−5q,+21,XY

Note: aCGH = array comparative genomic hybridization; MALBAC-NGS = multiple annealing and looping-based amplification cycle next-generation sequencing; PGD = preimplantation genetic diagnosis; PGS = preimplantation genetic screening; SNP = single-nucleotide polymorphism.

[a] Only parts of 2p and 2q were gained and there were still parts of the chromosome 2 that had a normal copy number.

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TABLE 2

Concordance analysis of MALBAC-NGS with aCGH.

Concordance analysis	Number
Chromosome calling comparison	720
Euploid chromosomes (true negatives)	674
Aneuploid chromosomes (true positives)	41
Missed chromosome calls (false negatives)	4
Extra chromosome calls (false positives)	1
Aneuploidy call performance[a]	
Sensitivity	91.11 (79.27–96.49)
Specificity	99.85 (99.17–99.97)
Positive predictive value	97.62 (87.68–99.58)
Negative predictive value	99.41 (98.49–99.77)

Note: aCGH = array comparative genomic hybridization; MALBAC-NGS = multiple annealing and looping-based amplification cycle next-generation sequencing.
[a] Values are percentage and 95% confidence interval.

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Robertsonian translocation, reciprocal translocation, or inversions, or from couples with advanced maternal age, recurrent pregnancy loss, or recurrent implantation failure (3, 16). The high abnormality rate is one of the major reasons for the low IVF success rate in these populations.

Previous studies using polar bodies to infer the chromosomal status showed a chromosomal aneuploidy rate of 22% to 72% in the oocytes (17). However, polar body biopsy only reflects the chromosome status from the maternal contribution and cannot guarantee a normal karyotype in the implanted embryos. Cleavage-stage biopsy reflects both the paternal and maternal contribution of the embryos, but the chromosomal aneuploidy rate can be as high as 30% to 85% (18, 19), bringing difficulties for implantation. Our previous study also showed only 26.09% of cleavage embryos were euploid (10). Although the aneuploidy rate of blastocysts is lower than that of cleavage embryos, the phenomenon of aneuploidy still exists in the blastocyst stage. Therefore, PGS is presently increasing to improve the pregnancy results in IVF cycles.

Recent technological developments in the area of WGA, microarray and NGS have allowed screening for chromosomal abnormalities along the whole genome. Both aCGH and SNP arrays have been used for PGS/PGD for several years, and MALBAC-NGS is a relatively new technology to the IVF community. Since the introduction of MALBAC WGA-NGS method in December 2012 (7), studies have been published on successful single-cell WGA and sequencing of a culture cell line (7), single human sperm (9), and single human oocytes (8), with detailed comparisons on technical and biological replicates to confirm the detection accuracy and reproducibility of the method (20–22).

TABLE 3

Concordance analysis of MALBAC-NGS with SNP array.

Concordance analysis	Number
Chromosome calling comparison	720
Euploid chromosomes (true negatives)	676
Aneuploid chromosomes (true positives)	41
Missed chromosome calls (false negatives)	2
Extra chromosome calls (false positives)	1
Aneuploidy call performance[a]	
Sensitivity	95.35 (85.54–98.72)
Specificity	99.85 (99.17–99.97)
Positive predictive value	97.62 (87.68–99.58)
Negative predictive value	99.71 (98.93–99.92)

Note: MALBAC-NGS = multiple annealing and looping-based amplification cycle next-generation sequencing; SNP = single-nucleotide polymorphism.
[a] Values are percentage and 95% confidence interval.

Huang. Validation of MALBAC-NGS for blastocysts. Fertil Steril 2016.

The MALBAC-NGS method was previously validated in our center using lysed and mixed genome contents from eight human fibroblasts as well as blastomere-stage embryos for PGD/PGS (10). Because blastocyst-stage PGD/PGS shows less embryo invasiveness and better effectiveness compared with cleavage-stage PGD/PGS, and it has been adopted by more and more centers in the practice of PGD/PGS (12), validation of the MALBAC-NGS method in blastocyst-stage PGD/PGS is therefore needed.

Next generation sequencing-based protocols have been validated in the PGD/PGS field. Fiorentino et al. (6, 23) used single cells from amniotic fluid as well as biopsies from cleavage-stage and blastocyst stage embryos for validation by comparing NGS and aCGH results. In our study, we used 30 blastocysts previously diagnosed with chromosomal abnormality. We compared three different methods: aCGH, SNP array, and MALBAC-NGS. In the 720 detected chromosomes, the consistent rate of MALBAC-NGS versus aCGH and SNP array reaches 99.31% (715 of 720) and 99.58% (717 of 720), respectively. The high consistency rate of MALBAC-NGS versus SNP array and aCGH indicates high accuracy in 24-chromosome screening using the current protocol.

The high consistent rates of the three methods also indicate a much lower chromosomal mosaicism rate in blastocyst embryos compared with cleavage-stage embryos. In our study, 86.67% (26 of 30) blastocysts showed identical results in all three detection methods. However, in our previous study, only 34.78% (8 of 23) cleavage embryos showed identical results in all three detection methods (10). We note that each method used a separate trophectoderm-cell biopsy, thus reflecting the differences of different trophectoderm cells from the sample blastocyst. Besides the lower mosaicism rate, blastocyst biopsy uses 3 to 6 cells and obtained more consistent WGA and chromosomal analysis results (24, 25). Trophectoderm cells biopsy also showed better biosafety compared with cleavage-stage biopsy. Scott et al. (26) showed that implantation rate of the embryo reduces 39% by performing cleavage-stage biopsy, whereas no major reduction was observed in the implantation rate from trophectoderm biopsying.

Although each blastocyst was examined by three methods, and each method used a separate trophectoderm-cell biopsy, we analyzed the concordance of MALBAC-NGS with aCGH and SNP arrays. When compared with aCGH, MALBAC-NGS specificity for aneuploidy call was 99.85% (674 of 675; 95% CI, 99.17–99.97), and the sensitivity was 91.11% (41 of 45; 95% CI, 79.27–96.49). When compared with SNP array, MALBAC-NGS specificity for aneuploidy

call was 99.85% (676 of 677; 95% CI, 99.17–99.97), and sensitivity was 95.35% (41 of 43; 95% CI, 85.54–98.72). The specificity is the proportion of embryos with a euploid aCGH/SNP array result that have a euploid NGS result. The sensitivity is the proportion of embryos with an aneuploid aCGH/SNP array result that have an aneuploid NGS result.

Fiorentino et al. (23) clarified the sensitivity (100%) and specificity (99.98%) of NGS compared with aCGH. There were some different details compared with our study. In their study, all the blastocysts were obtained from clinical PGS cycles, including euploid and aneuploid blastocysts. Moreover, they performed aCGH and NGS with the WGA product from the same trophectoderm cell sample. In our current study, only the blastocysts with chromosomal abnormalities detected by aCGH were recruited. The euploid blastocysts were either implanted into the uterus or frozen for further implantation. For this reason, we did not include euploid embryos in our concordance study. Furthermore, each method in our study used a separate trophectoderm cell biopsy, reflecting the different trophectoderm cells from the sample blastocyst.

In conclusion, MALBAC-NGS provides concordant chromosomal results versus aCGH and SNP array in blastocysts with chromosomal abnormalities. The current protocol for MALBAC-NGS requires only ~0.04x sequencing depth for obtaining a satisfactory 24-chromosome screening result. With the rapid development of the throughput and speed of NGS, hundreds of samples can now be pooled together with sequencing barcodes in a single sequencing run. We note that such technical advancement helps to significantly reduce the PGD/PGS cost while retaining a high level of detection accuracy compared with the existing techniques, which is essential for PGS/PGD to be widely adopted.

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