ORIGINAL ARTICLE: GENETICS

Validation of multiple annealing and looping-based amplification cycle sequencing for 24-chromosome aneuploidy screening of cleavage-stage embryos

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Objective: To validate multiple annealing and looping-based amplification cycle (MALBAC) sequencing for 24-chromosome aneuploidy screening of cleavage embryos and to explore the chromosomal characteristics of embryos at the cleavage stage.
Design: The 24-chromosome aneuploidy analyses of the blastomeres included comparative genomic hybridization (CGH), single nucleotide polymorphism (SNP), and MALBAC sequencing.
Setting: University-affiliated IVF center.
Patient(s): Three couples who delivered babies from the same IVF cycle, which included 23 donated, frozen cleavage embryos.
Intervention(s): None.
Main Outcome Measure(s): Three blastomeres were selected from each single embryo and subject to CGH, SNP, and MALBAC sequencing for 24-chromosome aneuploidy, respectively. The results of MALBAC sequencing were compared with the results of CGH and SNP. The chromosomal status and occurrence of the abnormal chromosomes were investigated. The relationship between the embryos' morphology and the euploid state was analyzed.
Result(s): Among the 23 donated embryos, the MALBAC sequencing results of 18 (78.26%) embryos were identical to those of CGH or SNP, including 8 embryos that had identical results by all three techniques. In terms of euploidy, only 6 of these 23 embryos (26.09%) were diploid. Blastomere abnormality was observed in all autosomes and sex chromosomes. In addition, the frequency of abnormality was different for certain chromosomes.
Conclusion(s): With sequencing at 0.04× genome depth, MALBAC sequencing has been validated as a satisfactory method for 24-chromosome aneuploidy screening. The proportion of abnormal chromosomes was high in cleavage-stage embryos, and any chromosome could be abnormal. (Fertil Steril® 2014;■:■–■. ©2014 by American Society for Reproductive Medicine.)
Key Words: Cleavage embryo, 24-chromosome aneuploidy screening, comparative genomic hybridization (CGH), single nucleotide polymorphism (SNP), multiple annealing and looping-based amplification cycles (MALBAC)

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Although IVF technology has rapidly evolved, and the quantity and quality of the retrieved eggs and embryos continue to improve, the embryo utilization rate is still low. Chromosome abnormality is one of the main causes of the low utilization rate of embryos. Several technologies

were applied for chromosomal screening of the embryos, and many studies have shown that preimplantation genetic screening (PGS) for aneuploidy increases the probability of successful IVF pregnancies (1–3). Fluorescence in situ hybridization has been used to test the embryonic chromosomes for more than 10 years (4, 5). However, fluorescence in situ hybridization can only be used to test 5–10 chromosomes in one embryo. In addition, fluorescence in situ hybridization requires that cells are fixed onto a microscope slide, which is a critical step that requires skill and experience. Recently, high-resolution methods for the complete karyotyping of a cell have been applied to PGS for aneuploidy. With whole-genome amplification (WGA), comparative genomic hybridization (CGH), single nucleotide polymorphism (SNP), and next-generation sequencing (NGS) are technologies that remedy the limitations of fluorescence in situ hybridization. All three of these techniques (CGH, SNP, NGS) can be used to analyze aneuploidy involving any of the 24 chromosomes (22 autosomes and the X and Y sex chromosomes). Comparative genomic hybridization and SNP are indirect DNA-based tests. Comparative genomic hybridization is an approach to investigate whole-chromosome copy number ploidy for PGS, whereas SNP detects the whole-chromosome SNPs. Treff and his colleagues (6) investigated the applicability of NGS to preimplantation genetic diagnosis and PGS. Their study evaluated the semiconductor-based NGS for genetic analysis of human embryos. Multiple annealing and looping-based amplification cycles (MALBAC) is a newly developed amplification method. Hou et al. (7), Lu et al. (8), and Zong et al. (9) and their colleagues introduced MALBAC for the genome analysis of single human oocytes and single human sperm cells. In the present study, we evaluated the practicality of MALBAC sequencing for 24-chromosome aneuploidy screening and compared its use with the established CGH and SNP methods.

Although PGS detects aneuploidy in embryos, most studies only focus on a single embryonic blastomere or several trophectoderm cells. This does not fully reflect the whole status of the embryonic chromosomes. In the present study, we describe the chromosomal status of cleavage embryos that had a normal morphology and were donated by IVF couples. Chromosomal analyses of three blastomeres from each embryo were performed by CGH, SNP, and MALBAC.

MATERIALS AND METHODS

This study was approved by the institutional review board of Peking University Third Hospital. We obtained written informed consent from every participant before the treatment.

All embryos used in this study were surplus to the IVF patients' needs. Three couples, who successfully delivered babies from the same IVF cycle, donated a total of 26 frozen, cleavage-stage embryos. The age of the women was less than 35 years. General information about the three couples is summarized in Table 1. All of the embryos were inseminated by intracytoplasmic sperm injection (ICSI) and slowly cryopreserved on day 3. The cryopreservation criteria were as follows: the embryos have more than four blastomeres and less than 50% debris. The 26 frozen embryos were thawed, their morphology was recorded, and the zona pellucida (ZP) was removed. Blastomeres were disaggregated by gentle pipetting. From each embryo, we selected three blastomeres with a clear nucleus. Each embryo's blastomeres were subjected to single-cell CGH, SNP, and MALBAC.

TABLE 1

Data on the donated couples.

Patient	Age of woman	Cause of infertility	No. of donated embryos	No. of donated embryos that had results from 3 blastomeres
A	29	Male factor	12	10
B	33	Male factor	7	7
C	32	Male factor	7	6

Huang. MALBAC sequencing of the embryos. Fertil Steril 2014.

Comparative genomic hybridization was performed on 24sure-plus chips (Bluegenome). The SurePlex DNA amplification system was used for WGA. Samples and control DNA (8 μL for each) were labeled with Cy3 and Cy5 fluorophores. The labeling time was approximately 3 hours with DNA resuspended in dexsulfate hybridization buffer and hybridized overnight under cover slides. After washing, a vacuum centrifuge was used to dry the microarray slides, followed by laser scanning. Blue Fuse software was used to analyze microarray data concerning chromatin loss/gain across all 24 chromosomes.

Single nucleotide polymorphism was performed on Human CytoSNP-12V2.1 chips (Illumina). The biopsied cells were lysed using an alkaline denaturation buffer (0.2 M NaOH) followed by a 4-hour modified multiple displacement amplification protocol using phi 29 polymerase to generate template DNA. The DNA product (4 μL; 200 ng) then underwent a 13-hour WGA protocol again using phi 29 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 (Illumnia) and allowed to hybridize for 12 hours. Each CytoSNP-12 bead chip contained approximately 301,000 SNPs and other genetic markers. Stringency washes were performed to remove unhybridized and nonspecifically bound DNA. The bead chips were dried in a desiccator and scanned using an Illumina iScan Bead Array Reader. Raw data analysis was accomplished using Illumina Genome Studio software (10).

We used MALBAC to amplify the DNA of single blastomeres as the third technology. The amplification was initiated with a pool of random primers, each of which have a common 27-nucleotide sequence and 8 variable nucleotides. Then, MALBAC was performed as described before (9). MALBAC can generate the micrograms of DNA required for NGS. Using an Illumina HiSeq 2000 platform, the amplified genome of each single blastomere was sequenced at approximately 0.04× genome depth. Therefore, 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 fulfills the standard for chromosomal copy number variation screening (>0.01× genome depth) and yields reproducible copy number variation results with approximately 1 Mb of resolution to detect the variation (7).

RESULTS

Among the 26 embryos, 23 had detectable results from all three blastomeres. Our article provides an in-depth discussion on these 23 embryos.

Consistency of MALBAC Sequencing with CGH or SNP Arrays

Among the 23 embryos, the MALBAC chromosomal sequencing results of 8 (34.78%, 8/23) were identical to the results with CGH and SNP arrays. The MALBAC sequencing results of 10 embryos (43.48%, 10/23) were identical to the chromosomal results with the CGH or SNP methods. In all, the MALBAC sequencing results of 18 embryos (78.26%, 18/23) were identical to results with CGH or SNP. However, there were two embryos that had identical results by CGH and SNP, but different results by MALBAC sequencing. In addition, there were three embryos that had different chromosomal results using all three techniques (Table 2).

Embryonic Euploidy and Uniformity in Sex Chromosomes

Among the 23 embryos, 8 embryos had identical results for all three blastomeres. Six embryos (26.09%, 6/23) were uniformly diploid (embryos 7, 8, 9, 11, 16, and 17), two (8.7%, 2/23) were aneuploid (embryos 22 and 27). Embryo 22 had chromosome 22 monosomy, and embryo 27 had chromosome 14 monosomy.

Among the 23 embryos, 15 (65.22%, 15/23) were mosaic. Among these mosaic embryos, 11 (47.83%, 11/23) were mixtures of diploids and aneuploids (embryos 4, 5, 10, 12, 13, 14, 15, 19, 21, 24, and 26), three (13.04%, 3/23) were mixtures of aneuploids and aneuploids (embryos 6, 18, and 25), and one (4.35%, 1/23) was a mixture of sex chromosomes (embryo 20) (Fig. 1).

Of the mosaic embryos, two (embryos 6 and 25) contained complex abnormal chromosomes. These embryos had division disorders and low potentials for development and implantation. Among the 23 embryos, 18 embryos (78.26%, 18/23) had consistent sex results in all three blastomeres, three (13.04%, 3/23) had consistent sex results in two

TABLE 2

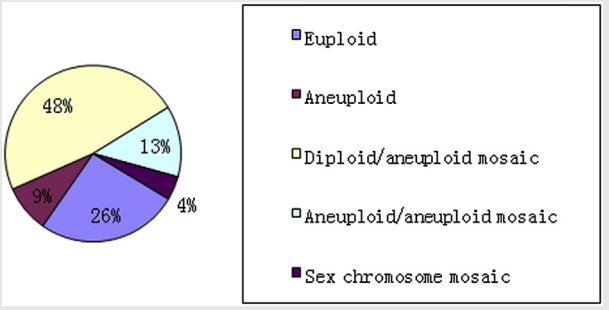
Morphology and the chromosomes of the cleavage embryos.

Patient	Embryo no.	High-grade embryo	SNP result	CGH result	MALBAC
Three blastomeres have the identical karyotype					
A	7	Yes	46,XY	46,XY	46,XY
	8	Yes	46,XY	46,XY	46,XY
	9	Yes	46,XY	46,XY	46,XY
	11	Yes	46,XX	46;XX	46;XX
B	16	No	46,XY	46,XY	46,XY
	17	No	46,XX	46,XX	46,XX
C	22	Yes	−22;XX	−22;XX	−22;XX
	27	Yes	−4q28,−14,XX	−4q;−14;XX	−4q;−14;XX
Two blastomeres have the identical karyotype					
A	4	No	46,XY	−15q;+16;+21;−22;XY	46,XY
	5	Yes	−17p,−20q,XX	46,XX	46,XX
	10	Yes	46,XY	+2;XY	+2;XY
	12	No	46,XX	46;XX	−3q,XX
	13	Yes	46,XX	+16;XX	+16;XX
B	14	No	46,XY	+13;XY	+13;XY
	15	No	−12q24.3,XX	46;XX	46;XX
	18	No	−20q13,46,XY	+1;−6p;−10;XY	+1;−6p;−10;XY
	20	Yes	46,XX	46,XX	46,XY
C	21	No	46,XX	+2q;+6p;−6q;−11q;XX	+2q;+6q;−6q;−11q;XX
	24	No	46,XY	−1;−2;−3;−7;−8;+4;+5; +6;+10;+12;+13;+14; +15;+20;+21;XY	46,XY
	26	No	46,XX	+2q;XX	46,XX
Each blastomere has a different karyotype					
A	6	Yes	46,XY	−1,−6,−15,+9,+10,+12, +13,+16,+18,19,+20,+21;XX	+18,XX
B	19	No	46,XX	−13q;XX	46,XY
C	25	Yes	12p+,−20,XY	−6;−15;−20;−21;−22;−X; +1;+2;+4;+16;+18;+Y	+1q,−3q,+4q,+6,+8q, +9q,+10q,−11q,−13q, −18q,−19q,−21,+22XX

Note: CGH = comparative genomic hybridization; SNP = single nucleotide polymorphism.

Huang. MALBAC sequencing of the embryos. Fertil Steril 2014.

FIGURE 1



Mosaic of the cleavage embryos.

Huang. MALBAC sequencing of the embryos. Fertil Steril 2014.

blastomeres, and only one embryo (4.35%, 1/23) had different sex chromosomes in all three blastomeres.

Relationship between Embryo Morphology and Chromosomes

The 23 embryos in this study were all at the D3 cleavage stage. The morphology of the embryos was more than four blastomeres and less than 50% debris before freezing. All of the embryos used in this study were available to transfer. In the present study, we defined the embryos that were at the six- to eight-cell stage, had uniform sizes, and contained $\leq$30% embryo debris as high-grade embryos. Among the 23 embryos, 12 were morphologically high-grade embryos, whereas 11 were not. Among the 12 excellent embryos, only 4 (33.33%, 4/12) were diploid.

Frequency of Abnormalities on Different Chromosomes

Among the 69 blastomeres in this study, 28 (40.58%, 28/69) were abnormal. The abnormalities occurred to all autosomes and sex chromosomes. However, the frequency of abnormality was different for different chromosomes (Fig. 2).

DISCUSSION

Chromosomal abnormalities may arise during germ cell and/or preimplantation embryo development. Many studies indicated a high chromosomal abnormality rate in IVF embryos (11–14). One reason is relevant to the formation of gametes, and 20%–50% of those cases are directly correlated to the maternal age; another reason is derived from the abnormality event after zygote formation, accounting for 30% of the total embryonic chromosomal abnormalities. The latter results from abnormal chromosomal separation during the embryonic divisions and is irrelevant of the maternal age. These abnormal separations might be associated with defects in the spindle. Because of the high proportion of defective oocytes in elderly women (15, 16), the selected three donors were aged less than 35 years, and all of them had healthy offspring with the same IVF cycle. The effects of the maternal age on embryonic abnormalities were consequently minimized.

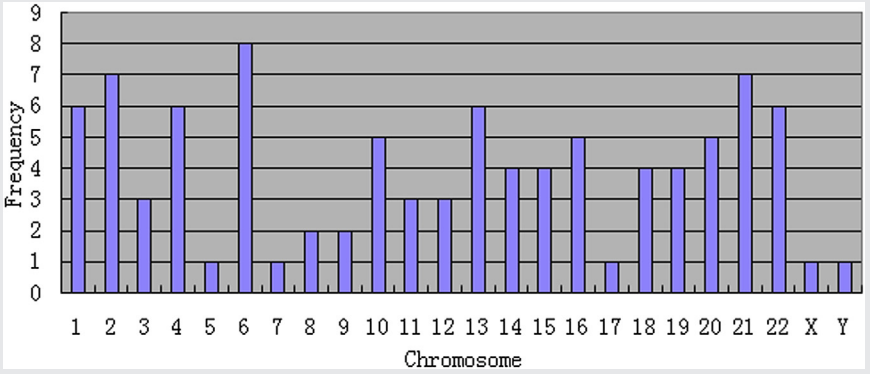
The embryonic chromosomes have direct impacts on embryo implantation and the successful development of those embryos into healthy babies. It is very difficult to comprehensively analyze the chromosomes using the conventional karyotyping technology because cleavage-stage embryos have only a few blastomeres. However, recent technical developments in the single-cell WGA, microarray, and sequencing have allowed chromosomal examinations to be carried out during the embryonic stage. The present study applied widely used clinical techniques including CGH, SNP arrays, and MALBAC sequencing. CGH and SNP have been used for PGS for several years, whereas MALBAC is a new technology from the NGS era. Since the first publication of the MALBAC WGA method in December 2012 (8), (9), other studies have been published using different systems: a cultured cell line (9), a single human sperm (8), single human oocytes (7), and circulating tumor cells (17). In three of these studies (7, 9, 17), detailed comparisons were carried out on technical and biological replicates to confirm the reproducibility of these systems.

In addition, we validated this system in our center. We lysed and mixed the genome contents of eight human fibroblasts and distributed the genomic DNA into eight MALBAC WGA reactions. All MALBAC single-cell-equivalent samples gave reproducible results. The purpose of the present study was to validate MALBAC sequencing for 24-chromosome aneuploidy screening of cleavage-stage embryos. Therefore, the amplified genome of each single blastomere was sequenced at 0.04$\times$ depth on a HiSeq 2000 platform, obtaining an average genome coverage of 3% for each single cell. Although these three techniques have their own characteristics, protocols, and costs, they can all successfully detect the chromosomes in a single cell (18). In the present study, three techniques were applied in parallel to detect the chromosomes of three blastomeres from the same embryo. As a result, the MALBAC sequencing results of 18 embryos (78.26%, 18/23) were identical to the results by CGH or SNP, including 8 embryos that had identical results using all three techniques.

Chromosomal mosaicism is high in cleavage-stage embryos. This is the main factor that leads to the inconsistent results among the three techniques. Only a few studies paid attention to the chromosomes of whole blastomeres from normally developing embryos. Mertzanidou et al. (11) investigated the chromosomal complements of 14 normally developing human cleavage-stage embryos using CGH. Their study shows that high-level mosaicism and structural aberrations are not restricted to arrested or poorly developing embryos but are common in good-quality IVF embryos.

Our study investigated the chromosomal status of 69 blastomeres from 23 donated embryos. Among these 23 embryos, the blastomere triplicates from 8 embryos had identical chromosomes, with 6 containing normal karyotypes and 2 containing abnormal karyotypes. Theoretically, those eight embryos (34.78%, 8/23) could lead to a successful pregnancy, which is consistent with the general

FIGURE 2



Frequency of the errors of individual chromosomes.

Huang. MALBAC sequencing of the embryos. Fertil Steril 2014.

cleavage embryo implantation rate (20%–25%) in IVF. Only six embryos (26.06%, 6/23) were diploid, whereas embryos with aneuploidy and mosaicism accounted for >70% of embryos. In the mosaic embryos, mixtures of diploidy and aneuploidy were most commonly observed; this mixture was observed in 11 embryos, accounting for 47.83% of the total embryos (11/23). The second most commonly observed mosaic embryos had mixtures of aneuploidy and aneuploidy; this group included three embryos and accounted for 13.04% of the total embryos (3/23).

In the present study, we found a high proportion of embryonic chromosomal mosaicism at the cleavage stage. Among the 23 embryos, 15 had mosaicism, accounting for 65.22% of the embryos (15/23), which is consistent with some previous results (19, 20). Among the 15 embryos containing chromosomal mosaicism, 12 displayed identical chromosomal results in 2 blastomeres. There are two developmental possibilities to explain these embryos. One possibility is mosaic embryos (i.e., the embryos would have a mixture of two cell lines). The other possibility is the marginalization of the cells with abnormal karyotypes, which acts as a self-correction process. During embryonic development into the blastocyst and later stages, abnormal cells are marginalized and excluded from the blastocyst so that only normal cells can develop into a fetus. Many studies have shown that embryos can self-correct during their development (21–24). Additional evidence for this is that fetuses with normal karyotypes are associated with abnormal placental cells. Among the 12 embryos with identical chromosomal results in 2 blastomeres, 7 contained identical normal karyotypes in 2 blastomeres, and 5 contained identical abnormal karyotypes in 2 blastomeres. These embryos had the potential to develop into blastocysts, resulting in successful implantation. Among the 15 embryos with chromosomal mosaicism, 3 had different results, even different genders, in all 3 blastomeres. Specifically, embryo numbers 6 and 25 presented complex abnormal chromosomes. It was postulated that these embryos had a division disorder and a low potential for development and implantation.

The Y chromosome is the smallest human chromosome. The X and Y chromosomes are homologous. However, their structures are different from those of autosomes. Regions of the X and Y chromosomes with identical structures are homologous, whereas regions with different structures are heterologous. Theoretically, a single blastomere contains two X chromosomes or one X chromosome and one Y chromosome. Therefore, the accuracy of sex tests at the single cell level has limitations. In embryo number 20, the autosomes were all normal by three methods; however, the sex chromosome results varied. Trophectoderm cells could be obtained through a blastocyst biopsy, which reduced the probability of the failed amplification of the single copy and increased the diagnostic accuracy of the sex chromosomes. In addition, the mosaicism rate in the blastocyst stage is lower than that in the cleavage stage. Therefore, a blastocyst biopsy is recommended in clinical PGS (25, 26).

At present, in most assisted reproductive laboratories, the selection of embryos is mainly based on morphological characteristics. Scholars realized that the embryos from the morphology-based selection have low developmental potentials. This is mainly because a large proportion of the embryos that are cultured in vitro contain abnormal chromosomes (27, 28). A recent study, which was based on the results from 6,000 embryos, showed that embryonic chromosomal abnormalities were also common to factors such as the maternal age and the embryonic morphology (27). For instance, even among the embryos with the best morphology from young mothers (aged <35 years), only 44% contained normal chromosomes. As the woman's age increases, the proportion of the embryos with abnormal chromosomes also increases. In the present study, the embryos that were at the six- to eight-cell stage, had uniform sizes, and contained ≤30% embryo debris were defined as high-grade embryos. Among the 23 embryos, 12 were morphologically high-grade embryos, whereas 11 were not. Among those 12 high-grade embryos, 4 had diploid chromosomes, only accounting for 33.33% (4/12); however, among the 11 "not high-grade" embryos, 2 displayed diploid chromosomes.

Humans have 23 pairs of chromosomes, with 22 pairs of autosomes and 1 pair of sex chromosomes. The present study showed that among 69 blastomeres, 28 (40.58%, 28/69) displayed abnormalities. Blastomere abnormalities were present at all autosomes and sex chromosomes; however, the abnormality frequencies were not identical on different chromosomes (Fig. 2).

Of course, the present study had limitations. The embryos in this study were frozen embryos that were donated by couples who underwent IVF and delivered babies. During the thawing process of those embryos, some blastomeres might be damaged. Therefore, we only selected three blastomeres with a clear nucleus from each embryo for the study. The chromosomal results of the embryos on the basis of three blastomeres could have some degrees of bias. Three methods, CGH, SNP, and MALBAC sequencing, were applied in this study to analyze the chromosomal content of three blastomeres from the same embryo. These three methods were technically mature; therefore, we did not take account for misdiagnosis introduced by the technical sensitivities. The selected 69 blastomeres derived from 23 embryos, which were all usable embryos in IVF, enabled the objective reflection of the chromosomal status of the embryos at the cleavage stage although the sample size was relatively small.

In summary, we used donated embryos from young couples to investigate the chromosomal status at the cleavage stage with three different techniques. With sequencing at 0.04× depth, MALBAC sequencing was validated as a satisfactory method for 24-chromosome aneuploidy screening. The results also showed that the proportions of abnormal chromosomes and chromosomal mosaicism were high in cleavage-stage embryos, which is an important factor in the low implantation rate at the cleavage stage. The accuracy of the diagnosis based on a single blastomere has limitations. Therefore, the cleavage stage is not the optimal stage for PGS. In addition, our results related to the embryonic morphology and chromosomal aberrations indicated that the morphological quality did not fully reflect the chromosomal content.

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