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ORIGINAL ARTICLE

Aneuploidy and sex concordance rate between cell-free DNA analysis from spent culture media of preimplantation embryo and DNA from whole embryo with respect to different morphological grading

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Abstract

Purpose: The aneuploidy and sex concordance between cell-free DNA in spent culture media (SCM) and DNA from whole embryo with respect to different morphological grading were examined to evaluate the feasibility of non-invasive preimplantation genetic testing for aneuploidy (niPGT-A).

Methods: A total of 46 pairs of embryos and corresponding SCM were divided into two groups based on the morphological grade. DNA was extracted from 22 and 24 pairs of low- and high-grade embryos, respectively, and respective SCM followed by chromosomal analysis using next-generation sequencing. Aneuploidy study and sex determination were conducted for both groups, and concordance rates were calculated.

Results: For low-grade embryos, 63.6% (14/22) were determined as aneuploidy by whole embryo analysis, and concordance rates were 54.5% (12/22) using niPGT-A. On the contrary, for high-grade embryos 41.7% (10/24) were determined as aneuploidy by whole embryo analysis, and concordance rates were 62.5% (15/24) using niPGT-A. The concordance rates were not statistically different between the low-grade and high-grade embryo groups ($p = 0.804$). For sex determination, concordance rates between whole embryo and SCM were 81.8% (18/22) and 87.5% (21/24) in low- and high-grade groups, respectively.

Conclusion: Aneuploidy and sex evaluation by niPGT-A may be feasible for both morphologically low- and high-grade embryos.

KEYWORDS
aneuploidy, low-grade embryos, next-generation sequencing, sex determination, spent culture media

1 | INTRODUCTION

In recent years, many studies on preimplantation genetic testing for aneuploidy (PGT-A) have progressed and contributed to the positive outcomes of assisted reproductive technology.[1–3] DNA extracted from TE cells obtained by blastocyst biopsy is amplified and analyzed using next-generation sequencing. This is followed by selection and preferential transplant of euploid embryos, which reduces the miscarriage rate expectedly. However, TE biopsy leads to certain detrimental effects in embryo quality.[4] For example, biopsy in early-stage embryos reduces the implantation rate compared with non-biopsy embryos. Therefore, morula or embryos in earlier developmental stages are not recommended for biopsy. Although implantation rate is not affected when biopsy is performed during blastocyst stage, its long-term effects are unclear. Moreover, TE biopsy is associated with certain techniques and high cost, which makes it a difficult procedure to be performed at general IVF clinics.[5]

For last one decade, non-invasive PGT-A (niPGT-A) has been actively studied as a new embryo evaluation method to solve these problems.[6–12] It was in 2013 that cell-free DNA in human blastocoele fluid was analyzed for genetic diagnosis of embryo in preimplantation stage.[13] In 2015, Wu et al.[14] demonstrated that autosomal-dominant mendelian disease could be diagnosed by using cell-free DNA from SCM. Later, cell-free DNA was analyzed for PGT-A using genome-wide study by microarray or next-generation sequencing.[15] To establish the clinical availability, the concordance rate of niPGT-A with TE biopsy or whole embryo was calculated repeatedly for years and the results varied from 30 to 100%.[16] The possible reasons for this wide range of concordance rate included differences in culture conditions, sampling time, and sequencing method. Although the non-invasive approach is expected to have great potential, the clinical method to use niPGT-A has not been determined yet.

Morphological classification of embryos based on the Gardner or Veeck score has been used as a conventional non-invasive embryo evaluation method.[17] Almost all IVF clinics have routinely evaluated the morphological grade of cultured embryos and used it as a reference for embryo selection and transfer. There is some evidence that a morphologically low-grade embryo is associated with higher aneuploidy rate than a high-grade embryo.[18,19] However, embryos with low-grade can be euploid and possess developmental competence. In some cases, even an embryo in early preimplantation stage, which is considered non-suitable for biopsy, may yield a healthy baby. Therefore, the morphological grading is not an absolute measure, and there is a potential risk to discard such morphologically low-grade but competent embryo.

Therefore, low-grade embryos should be subjected to PGT-A as well as high-grade embryos. In the present study, we utilized niPGT-A to study low-grade embryos and compared the results with those of high-grade embryos. To the best of our knowledge, this is the first study that examined the clinical utility of niPGT-A for using low-grade embryos that are considered incompetent otherwise.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

This study was deliberated and approved by the ethical committee of Chiba University Hospital (Permission number: 669) and The Japan Society of Obstetrics and Gynecology. All embryos and SCM were obtained from the patients treated with IVF after obtaining written informed consent.

2.2 | Patients background

A total of 33 patients aged from 27 to 44 years (mean age-36.5 years) from three IVF facilities, that is, Tsukuba University hospital (Laboratory 1), Kobanawa Medical Clinic (Laboratory 2), and Hara Medical Clinic (Laboratory 3) participated in the study. All patients offered to participate in the study consented to this study. After controlled ovarian stimulation, patients were approached for oocyte retrieval.

2.3 | IVF cycle

Controlled ovarian stimulation (COS) was performed individually by the attending physician according to the patient's age, hormone levels, ovarian reserve, status of previous cycles. Briefly, either long protocol, short protocol, antagonist protocol or minimal stimulation protocol was selected according to the patient's status. Folyrmon-P (Fuji Chemical Industrial), Gonapure (ASKA Pharmaceutical), Gonalef (Merck Biopharma), Ferring (Ferring Pharmaceuticals) or Clomid (Fuji Chemical Industrial) was used alone or in combination for ovarian stimulation. As ovulation triggers Ovidrel (Merck Biopharma), HCG Mochida (Mochida Pharmaceutical) or Suprecure (Clinigen) was administered at the appropriate timing evaluated from the follicle diameter and hormone status. Transvaginal oocyte retrieval was performed 34 h after ovulation trigger.

2.4 | Embryo culturing and sample collection

Cumulus oocyte complexes (COCs) were washed repeatedly by Multi handling medium (Irvine Scientific). COCs were transferred to Continuous Single Culture NX Complete (CSCM-NX) (Irvine Scientific) or ORIGIO Sequential medium (CooperSurgical Fertility Solutions) and inseminated with sperms (10×10^4/ml) as part of conventional IVF. For ICSI, COCs were denuded of gametes by addition of hyaluronidase, and matured oocytes were inseminated. Fertilization was confirmed by observing pronuclei after 18 h, and fertilized embryos were cultured separately in 50 µl of CSCM-NX. This was followed by medium change on 3rd day. Thereafter, on 6th or 7th day, embryos were cryopreserved, and SCM was collected and frozen at −20°C for PCR. The fertilized embryos were thawed and transferred into a PCR tube with minimum amount of washing medium.

2.5 | Assessment of embryo quality

On the morning of the last culture day, developing blastocysts were evaluated using Gardner's scoring system and divided into high- (>3 score, class A and B) and low-grade embryos (≤3 score, class C or below). Evaluation of embryos was performed by multiple embryologists at each laboratory. The embryos unable to reach the blastocyst stage were classified as low-grade embryos.

2.6 | DNA amplification and next-generation sequencing

For niPGT-A, frozen SCM was thawed at room temperature and used as template for DNA amplification. For the whole embryo analysis, corresponding embryo was thawed and transferred to 20 μl of nuclease free water. This resulted in bursting of the embryo and was followed by extraction of DNA. DNA samples (8 μl each) were used as templates for genome-wide DNA amplification by PG-Seq Rapid Non-Invasive Kit (PerkinElmer). This kit was based on the highly multiplex PCR method and can amplify genome-wide DNA. The 1st PCR for DNA amplification (95°C 3 min for initial denaturation, 8 cycles of 98°C 20 s, 25°C 1 min, 72°C 30 s followed by 16 cycles of 98°C 20 s, 58°C 1 min, 72°C 30 s) and 2nd PCR for index addition (95°C 3 min for initial denaturation, 4 cycles of 98°C 20 s, 50°C 30 s, 72°C 30 s followed by 6 cycles of 98°C 20 s, 62°C 30 s, 72°C 30 s) were conducted according to the manufacturers' protocol. After DNA extraction using magnetic beads, the purified library was evaluated by 4150 TapeStation (Agilent) for quality check, diluted to 80 pM, and sequenced by iSeq 100 system (illumina). NGS settings were bilateral reads at 250 cycles with dual index.

2.7 | Data processing

FASTQ files from each specimen were trimmed by Trimmomatic (USADEL LAB). Reads shorter than 50 bases were discarded and those with a quality score of <20 were trimmed. Purified reads were mapped to human genome (hg.19) by BWA and sorted by SAMTOOLS to make BAM file (Genome Research Limited). BAM files were analyzed by PG-FIND v2.0 (Perkinelmer) to depict chromosomal graph for aneuploidy and sex analysis. Default threshold was used for aneuploidy call. For sex determination, an embryo with mapped reads beyond the threshold for Y chromosome was named as male (XY). All mapped graphs were shown in Figure S1.

2.8 | Statistical analysis

Statistical analysis was conducted by using R software v3.5.0 (R Foundation for Statistical Computing). The χ^2 test was used to analyze statistical difference for aneuploidy rate or concordance rate; p-values of <0.05 were considered as statistically significant.

3 | RESULTS

Of 46 embryos donated by 33 patients, 22 and 24 embryos were classified as low-grade and high-grade embryos, respectively based on morphological classification by the Gardner or Veeck score. The age of patients was not different between the two quality groups (average ± S.D; 37.3 ± 4.64 vs. 36.2 ± 4.83 for low- vs. high-grade groups, respectively). The ages of patients and morphological grades of corresponding embryos in each group are summarized in Table 1 (low-grade) and Table 2 (high-grade).

Karyotype analysis by NGS using whole embryo showed that 64% (14/22) of low-grade and 42% (10/24) of high-grade embryos were called aneuploid. Although aneuploidy rates appear higher in low grade embryos, the difference was statistically non-significant between the two groups ($p = 0.136$).

In all SCM samples, sequencing was found to be successful for the determination of karyotype, which was compared with the corresponding whole embryo sequencing. It was thus possible to compare the results of whole embryos and corresponding SCM in all specimens. The embryos for which both SCM and whole embryo sequencing results detected identical ploidy of all chromosomes, were classified as "fully concordant" (shown by ++ in Tables 1 and 2), both SCM and whole embryo sequencing results detected the same aneuploidy of ≥1 chromosome, were classified as "partially concordant" (shown by + in Tables 1 and 2), and either SCM or whole embryo sequencing results detected euploid and other as aneuploid, the embryos were classified as "dis-concordant" (shown by − in Tables 1 and 2). For example, a sample of T02 in low-grade embryos showed a karyotype of 45, −10, XY obtained both from the whole embryo and SCM (Table 1). Thus, karyotype evaluation was completely concordant and T02 was judged to be "full concordant (++)." Whereas, a sample of T11 showed 48, +3, +13, XY evaluated from the whole embryo although the result of 47, +3, XY was obtained from SCM analysis. Examples such as T11 were judged to be "partial concordant (+)" because only part of the aneuploidy matched.

Within the low-grade embryos, 36% (8/22), 18% (4/22), and 45% (10/22) of karyotype results analyzed by whole embryo and SCM were fully concordant, partially concordant, and dis-concordant, respectively (Table 1). On the other hand, within the high-grade embryos, 54% (13/24), 8% (2/24), and 38% (9/24) were fully concordant, partially concordant, and dis-concordant, respectively (Table 2). The fully concordant rates were not significantly different between the two groups ($p = 0.23$). Because full concordance between SCM and whole embryo results are essential for clinical application, sensitivity and specificity were calculated for full concordance. The sensitivity represents the true positive rate for aneuploidy and it was 62.5% (5/8) and 78.6% (11/14) for low- and high-grade groups, respectively. The specificity represents the true negative rate for aneuploidy and it was 64.3% (9/14) and 60% (6/10) for low- and high-grade groups, respectively.

We independently analyzed the concordance rate of the sex chromosomes because it differs from the autosomal determination method. Sex determination was also conducted using SCM sequencing and compared with the results of whole embryo sequencing.

TABLE 1 Concordance of aneuploidy and sex analysis for low-grade embryo and corresponding SCM.

Laboratory ID	Blastocyst ID	Patient ID	Age	Grade	Karyotype		Autosome concordance	Sex concordance
					Embryo	SCM		
Laboratory 1	T01	001	39	3CC	47, +12, XY	46, XX	–	–
	T02	001	39	3BC	45, −10, XY	45, −10, XY	++	+
	T03	001	39	2C	46, XY	46, XX	++	–
	T04	002	39	3CC	45, −16, XY	45, −16, XY	++	+
	T05	003	39	4CC	46, +10, −12, XX	46, XX	–	+
	T08	004	27	1C	46, XX	45, −9, XY	–	–
	T10	005	37	2B	46, XY	46, XY	++	+
	T11	005	37	4CB	48, +3, +13, XY	47, +3, XY	+	+
	T12	005	37	4CB	45, −16, XY	45, −16, XY	++	+
	T13	006	32	4CB	46, XY	46, XY	++	+
	T15	007	44	1B	45, −2, +10, −18, XY	46, XY	–	+
	T16	008	40	4CB	47, +15, XX	46, −2, +15, XX	+	+
Laboratory 2	K01	009	32	3CB	46, +4, +5, +6, −17, −19, −12, XY	47, +16, XY	–	+
	K02	010	41	4BC	45, −16, XY	44, −16, −21, XY	+	+
	K05	011	40	3AC	46, XX	46, −1, +20, XX	–	+
Laboratory 3	H01	012	45	G4	43, −1, +5, −8, −15, +16, −17, XY	47, +5, XY	–	+
	H02	013	41	early BL	48, +16, +18, XX	46, XX	–	+
	H03	014	36	G4	46, XX	46, XX	++	+
	H04	015	39	early com	46, +2, +6, −20, −22, XX	46, XX	–	+
	H05	016	28	early com	46, XX	45, −3, XX	–	+
	H06	017	39	4CC	46, +10, −18, XY	45, −4, +10, +13, −16, −17, −18, +20, XX	+	–
	H07	018	37	Morula	46, XX	46, XX	++	+

Note: ++: Full concordance between embryo and SCM with respect to the number of aneuploid chromosomes. +: Partial concordance between embryo and SCM with respect to the number of aneuploid chromosomes.

Abbreviations: early BL, early-stage blastocyst; early com, early compaction.

However, sex was determined only by whether the Y-chromosomal read exceeded a threshold. The concordance rates were 81.8% (18/22) and 87.5% (21/24) for the low- and high-grade groups, respectively (Tables 1 and 2). The concordance rates were not significantly different between the two groups ($p = 0.592$).

4 | DISCUSSION

In the present study, cell-free DNA in SCM was used as template for aneuploidy analysis and sex determination by next-generation sequencing-based niPGT-A. The concordance rates were compared with the results obtained from corresponding whole embryo sequencing. These results were divided into two groups according to the morphological grading of embryos mainly by Gardner's scoring system to pursue clinical availability of niPGT-A, especially for low-grade embryos.

It is clear that the morphological grading is an important factor in evaluation of embryos and sometimes it is the only factor considered at general IVF clinics for the embryo selection and further transplant. Minasi et al. demonstrated that the aneuploidy rate is clearly increased at all ages with a decrease in the morphological

TABLE 2 Concordance of aneuploidy and sex analysis for high-grade embryo and corresponding SCM.

Laboratory ID	Blastocyst ID	Patient ID	Age	Grade	Karyotype		Autosome concordance	Sex concordance
					Embryo	SCM		
Laboratory 1	T06	019	33	4BB	48, +2, +18, XX	46, XX	−	+
	T07	004	27	4AB	46, XX	45, −8, XX	−	+
	T09	005	37	4BA	45, −22, XY	45, −22, XY	++	+
	T14	020	42	4BB	48, −8, +9, +11, +16, +21, −22, XX	47, −8, +9, +11, +16, −22, XX	+	+
Laboratory 2	K03	021	33	5BB	46, XY	46, XY	++	+
	K04	022	39	4AB	46, XX	48, +4, +15, XX	−	+
	K06	023	35	3AB	46, XX	46, XX	++	+
	K07	024	35	4BB	49, +13, +19, +21, XY	47, +13, XY	+	+
	K08	025	31	4BB	46, XX	46, XX	++	+
	K09	026	33	4AB	45, −15, XY	48, −4, +14, +17, +22, XY	−	+
	K10	027	41	4BB	46, XY	46, XY	++	+
	K11	028	30	4AA	47, +15, XY	46, XX	−	−
	K12	029	42	3BB	45, −16, XY	46, XX	−	−
	K13	030	36	4BA	46, XX	48, +1, +22, XY	−	−
	K14	031	25	4BB	47, +12, XY	47, +19, XY	−	+
	K15	032	38	4BB	45, −17, XY	46, XY	−	+
Laboratory 3	H08	033	37	4BB	48, +8, +14, XY	48, +8, +14, XY	++	+
	H09	033	37	4BB	45, −22, XY	45, −22, XY	++	+
	H10	033	37	4BB	46, XY	46, XY	++	+
	H11	033	37	4AB	46, XY	46, XY	++	+
	H12	033	37	4BB	46, XY	46, XY	++	+
	H13	033	37	4BB	46, XY	46, XY	++	+
	H14	033	37	4BB	46, XX	46, XX	++	+
	H15	033	37	4BB	45, −4, XY	45, −4, XY	++	+

Note: ++: Full concordance between embryo and SCM with respect to the number of aneuploid chromosomes. +: Partial concordance between embryo and SCM with respect to the number of aneuploid chromosomes.

grading of ICM or TE.[18] Nevertheless, a certain number of embryos with low morphological grading are euploid and have potential to develop into a healthy baby. For example, approximately 30% of the blastocysts with C score of either ICM or TE were euploids. Such morphologically low-grade embryos were otherwise discarded by the general IVF clinics with an assumption of higher aneuploidy rate. Our results showed that roughly two thirds of low-grade embryos and one third of high-grade embryos were aneuploids when whole embryos were used as a specimen. These findings also demonstrate that embryos that have a potential for a baby had been disposed. For example, we demonstrated that the morula from 37-year-old patient (H07 in Table 1) was found to be euploid.

The concordance rates for aneuploidy analysis of niPGT-A and whole embryo have been studied repeatedly and summarized in several reviews.[5,16] A very wide range of concordance rates were reported, with some papers reporting about 50% concordance and others reporting almost 100% concordance. The underlying reason for this wide range of concordance rates has been studied but not clearly elucidated. Patient background, insemination method, timing of medium change or medium type, extent of contamination, DNA amplification method, next-generation sequencing method, definition of concordance and the program used for data analyses are some possible factors resulting in this variation. Previous studies have shown that the concordance rates between the results obtained from TE biopsy and whole embryos ranged from 59.5%–89.7%.[20–22] Therefore, niPGT-A seems to be an optimal technique for evaluating the chromosomal status. Our results of niPGT-A showed the concordance rates of 54.5% and 62.5% in low- and high-grade embryos, respectively. The concordance rates were not statistically different between the two groups raising the possibility of niPGT-A as an optimal evaluating method, especially for low grade embryos. Considering clinical use of niPGT-A, it is necessary to increase the concordance rate with whole embryos to the same level as biopsy-based PGT-A.[21] However, as this study showed, niPGT-A seems to be a useful technique even at this stage for low-grade embryos that are expected to be damaged by biopsy. A candidate for improvement is the method used for DNA amplification. We used multiplex PCR-based method for DNA amplification; however, recent reports have shown that MALBAC-based whole genome amplification method was associated with higher concordance rates.[23,24]

The concordance rates of sex determination were analyzed exclusively in the present study as its threshold was different from the aneuploidy analysis. The sequence depth of only Y-chromosome needs to be exceeded by threshold for a DNA to be male (XY). This is different from the analysis of autosomal chromosomes where program needs to distinguish the sequence depth of 2:1 (monosomy) or 2:3 (trisomy). As a result, the concordance rates of sex determination between niPGT-A and whole embryo was more than 80% in both low- and high- grade embryos. This higher rate of concordance than aneuploidy analysis is important in the diagnosis of X-linked mendelian diseases such as hemophilia.[25] On the other hand, this higher concordance rate can be ethically controversial in terms of preimplantation sex selection. For example, in countries and regions where men and women are socially unequal, it may lead to an imbalance in the number of male and female births.

There are several limitations of our study. Firstly, the number of specimens used was small. The recent studies have reported the analyses of more than 500 embryos.[6] According to our data, the appropriate sample size (n) was calculated statistically under the condition of 5% significance level and the power as 0.90. As a result, sample size of more than 59 was appropriate ($n \geq 58.2$). The second limitation is associated with the clinical outcomes. In this study, the results obtained from whole embryo were compared with those of niPGT-A; however, it was not clear if the chromosomal status of implanted embryos was identical to that of the baby because of the mosaicism of cells and the possibility of recovery from aneuploidy during post-implantation development.

5 | CONCLUSION

The chromosomal status comparison between SCM and whole embryo demonstrated a practical concordance rate in morphologically low- and high-grade embryos. Higher concordance rate was observed for sex determination and may help in the diagnosis of X-linked Mendelian disease. With slight improvement in the concordance rate, niPGT-A using SCM may serve as an optimal evaluating method, especially for low-morphological-grade embryos, which are not suitable for biopsy and discarded otherwise.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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