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Preimplantation Genetic Testing of Aneuploidy by Next Generation Sequencing: Association of Maternal Age and Chromosomal Abnormalities of Blastocyst

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Abstract

BACKGROUND: Aneuploidy is a major cause of miscarriages and implantation failure. Preimplantation genetic testing for aneuploidy (PGT-A) by Next Generation Sequencing (NGS) is able to detect of the numeral and structural chromosomal abnormalities of embryos *in vitro* fertilization (IVF).

AIM: This study was aimed to assess the relationship between maternal age and chromosomal abnormalities NGS technology.

METHODS: A group of 603 human trophectoderm (TE) biopsied samples were tested by Veriseq kit of Illumina. The relation of marternal age and chromosomal abnormality of blastocyst embryo was evaluated.

RESULTS: Among the 603 TE samples, 247 samples (42.73%) presented as chromosomal abnormalities. The abnormalities occurred to almost chromosomes, and the most popular aneuploidy observed is 22. Aneuploidy rate from 0.87% in chromosome 11 to 6.06% in chromosome 22. The rate of abnormal chromosome increased dramatically in group of mother's ages over 37 (54.17%) comparing to group of mother's ages less than 37 (38.05%) (p < 0.000). The Abnormal chromosome and maternal age has a positive correlation with r = 0.4783 (p<0.0001).

CONCLUSION: These results showed high rate abnormal chromosome and correlated with advanced maternal age of blastocyst embryos.

Introduction

Maximizing the success rate of *in vitro* fertilization (IVF) treatments is challenge, and a reliable mean of determining the embryos with the most significant capacity for pregnancy is required [1]. There are several molecular methods to assess embryos, which are available to be applied. The major cause of IVF failure was showed to be abnormal chromosome. Recent years, aneuploidy rates were higher in oocytes and embryos from women of advanced maternal age [2], [3], [4]. The number of women intentionally, delaying pregnancy after year-old age of 35, has increased significantly last decades because the clash between the optimal biological period for women to have children and to obtain additional education, and building a career. In fact, for women over 40 years old, it is common for more than fifty percent of the oocytes retrieved to be aneuploidy [1], [5]. In miscarriage couples, an abnormal embryonic karyotype has been found to represent the most frequent cause [3].

PGT-A has been applied for the last over ten years to assess the chromosome abnormality of embryos to satiate the reproductive outcome of specific patient groups. Aneuploidies are common in early human embryos such as day 3 to day 5. Most methodologies of embryo assessment involve morphologic analysis at different developmental stages of the embryo. Blastomere number, multi-nucleation, fragmentation of embryo, and blastocyst formation are the key factors associated with viability

of embryo. The relationship between morphology and embryo aneuploidy was first evaluated with Fluorescence *in situ* hybridization (FISH) studies for 7–8 chromosomes [6]. Pellicer's experience showed that a remarkable rate of embryos with chromosomal abnormality was able to develop to the blastocyst with suitable morphology parameters; in fact, 42.8% of chromosomally abnormal embryos reached blastocyst stage [7]. However, the failure of finding a better morphologic indicator of aneuploidy in previous researches had been a consequence of technical insufficiency. Virtually all study has evaluated only a few specific chromosomes in each embryo, and it is therefore unavoidable that some of the embryos categorized as aneuploidy were, in fact, abnormal, with aneuploidies affecting chromosomes that were not tested [1].

More recently, in a study, array comparative genomic hybridization (A- CGH) could be used to detect 24 embryonic chromosomes. A-CGH was the first technology to be widely available for comprehensive aneuploidy screening and is now used extensively around the world [8]. The rapid development of next generation sequencing (NGS) technologies has generated an increasing interest to apply in PGT-A purposes and the technique offer improvements for the detection of chromosomal aneuploidy in IVF embryos compared with current technologies by reduced costs and enhanced precision as well as parallel and customizable analysis of multiple embryos in a single sequencing run [8], [9]. In this study, we applied NGS technology to screening 24-chromosome aneuploidy on embryos at blastocyst stages. This approach with trophectoderm samples from blastocyst biopsies, both whole chromosome aneuploidy and segmental chromosome imbalances would be detected. We also determined the chromosomal abnormalities of blastocyst-stage embryos *in vitro* fertilization using PGS-NGS technique and evaluating the correlation between the chromosomal status of blastocyst-stage embryos and the maternal age.

Materials and Methods

Material

The study population consisted of 603 embryos from consecutive patients planning to undergo PGS with trophectoderm (TE) biopsy. All IVF cycles were performed at the Andrology and Fertility Hospital of Hanoi, and Hanoi Hospital of Obstetrics and Gynecology in the period between June 2017 and February 2018. Genetic testing was performed at the DNA laboratory - Vietnam Military Medical Academy.

Whole genome amplification (WGA)

For whole genome amplification, TE samples, negative and positive controls were lysed, and genomic DNA was amplified by SurePlex kit (Illumina, Inc., San Diego, CA, USA), as the manufacturer's recommendation. Then, 5 µl of each product plus 5 µl gel loading dye were tested by agarose electrophoresis to determine the success of the amplification.

Libraries preparation

Libraries were prepared at DNA Lab using the VeriSeq PGS workflow (Illumina, Inc.), was briefly following: One nanogram of quantified dsDNA template at 0.2 ng/ml was added to 5 µl of Amplicon Tagmentation Mixture (ATM) and 10 µl of Tagmentation DNA Buffer (TD). The segmentation step was carried out at 55^oC for 5 min and hold at 10^oC. The resulting segmented mixture was neutralized by adding 5µl of proprietary neutralization buffer (NT). Post-homogenization, the Tagmentation plate was held at room temperature for 5 min. The fragmented DNA was amplified via a limited-cycle PCR programme (one cycle of 72^oC for 3 min, followed by 12 cycles of 95^oC for 10 s, 55^oC for 30 s and 72^oC for 30 s, one cycle at 72^oC for 30 s, followed by a hold at 4^oC) after the adding of 5µl of index 1 (i7), 5µl of index 2 (i5), and 15µl of Nextera PCR Master Mix (NPM) to each well. PCR product clean-up used AMPureXP beads (Beckman Coulter, Brea, CA, USA) to purify the library DNA with no salt carryover, providing a size selection step that removes short library fragments including index 1 (i7) and index 2 (i5) from the population. 45 µl of the PCR product was transferred to 96-well storage plates containing 45 µl of AMPure XP beads. Sealed plates were mixed using a microplate shaker at 1000 rpm for 2 min, then incubated at ambient temperature without shaking for 5 min. Thereafter, the plate was placed on a magnetic stand for 2 min or until the supernatant cleared. While the plates were kept on the magnetic stand, the magnetic beads were washed twice with 200 µl of freshly prepared 80% ethanol. Purified libraries were eluted with 50 µl of the Nextera XT Resuspension Buffer. It could be found more detail elsewhere [8], [9].

Sequencing

Single-end, dual index 36 base pair reads sequencing was performed at DNA Laboratory following the Illumina chemistry workflow on a MiSeq (Illumina, Inc.), using the MiSeq Reagent Kit v3 PGS kit (Illumina, Inc.) which contains the ready to load onboard clustering and SBS chemistry reagents.

Processing and analysing data

The following bioinformatics analysis was accomplished with a pre-release version of BlueFuse Multi for NGS (Illumina, Inc.). "Embryos were diagnosed as "euploidy" if the generated plot showed no gain or loss [8]. Secondary analysis included statistics; description, processing, and data analysis were performed by STATA software version 14. We compared the average values by t-testst and the

ratios by Chi quare test, CI 95%. We use simple linear regression and Pearson's correlation to find the relationship.

Results

Of the blastocyst-stage embryos, 95.9% were succeeding in whole genome amplification (578 out of 603). We investigated 410 embryos belong group mother's age <37 years old Group 1 (32.6 ± 3.43) and 168 embryos belong group mother's age >37 years old – Group 2 (39.63 ± 2.96) (Table 1).

Table 1: PGS-NGS results in 578 embryos at blastocyst stage, (Pearson chi2(1) = 12.6506 pr = 0.000)

	Group 1 (<37 years old)	Group 2 (≥37 years old)	Total	P (Group 1 vs Group 2)
Average age (Mean±SD)	32.06 ± 3.43	39.63 ± 2.96	34.26 ± 4.76	0.0000
Normal embryos (n, %)	254 61.95%	77 45.83%	331 57.27%	0.0000
Abnormal embryo (n, %)	156 38.05%	91 54.17%	247 42.73%	0.0000
Total embryo (n, %)	410 100%	168 100%	578 100%	

Note: P values were determined by t test and Chi square test.

A wide variety of aneuploidies was detected. Indeed, the results showed that any chromosome can be affected by aneuploidy at the blastocyst stage. A total of 322 chromosomal abnormalities were detected in the aneuploidy embryos represented monosomy, trisomy (Figure 1). Top 3 chromosomal 22, 16 and 21 were much more represented by abnormal than others (35, 32 and 24, respectively) (Table 2).

Table 2: Characteristics of aneuploidy

Chromosome	Total chromosome tested	Aneuplody n	%	Euploidy n	%
1	578	9	1.56%	569	98.44%
2	578	11	1.90%	567	98.10%
3	578	12	2.08%	566	97.92%
4	578	15	2.60%	563	97.40%
5	578	13	2.25%	565	97.75%
6	578	9	1.56%	569	98.44%
7	578	7	1.21%	571	98.79%
8	578	17	2.94%	561	97.06%
9	578	6	1.04%	572	98.96%
10	578	17	2.94%	561	97.06%
11	578	5	0.87%	573	99.13%
12	578	12	2.08%	566	97.92%
13	578	15	2.60%	563	97.40%
14	578	10	1.73%	568	98.27%
15	578	19	3.29%	559	96.71%
16	578	32	5.54%	546	94.46%
17	578	10	1.73%	568	98.27%
18	578	11	1.90%	567	98.10%
19	578	7	1.21%	571	98.79%
20	578	8	1.38%	570	98.62%
21	578	24	4.15%	554	95.85%
22	578	35	6.06%	543	93.94%
XY	578	18	3.11%	560	96.89%
Total	13294	322	2.42%	12972	97.58%

As expected, a strong association between advancing maternal age and aneuploidy was observed, but interestingly individual chromosomes were not equally affected by this phenomenon. There were 54.1% embryos had chromosomal abnormality in group 1 whereas only 38.05% in Group 1 (Table 1).
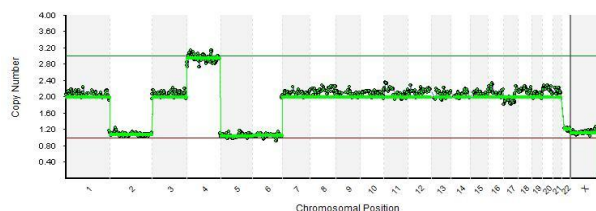

Figure 1: Examples of chromosomal abnormalities detection by next-generation sequencing. Embryo number HU3 of patient N. T. H.: 42, XY, -2, -5, -6, -22, +4

Results from the embryos of group 1 were compared with those from patients in group 2. Chromosome 14 showed the greatest increase in the risk of aneuploidy (3.7-fold increase). Chromosomes 7 and 18 displayed a 3.3 and a 2.9-fold increase, respectively. For chromosomes 2, 3, 12, 13, 15, 17, 19, 20, 21 and 22 the increase was around from 1.5 to 2-fold. Other chromosomes were found little or no change in aneuploidy rate with advancing age (Figure 2).
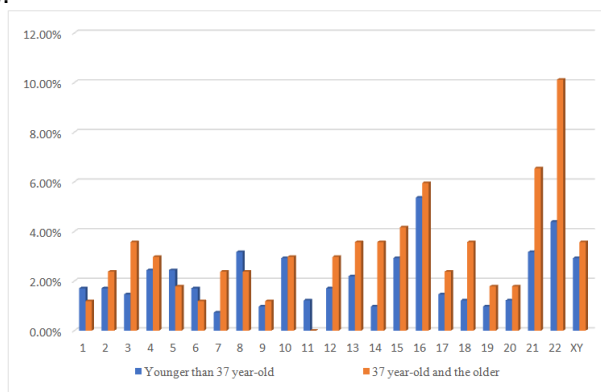

Figure 2: Incidence of chromosomal abnormalities between two groups

We evaluated the relationship between blastocyst-stage chromosomal abnormalities and maternal age using linear regression and Pearson's correlation (Figure 3). The correlation coefficients r = 0.4783 (CI 95%) are illustrated as formula:

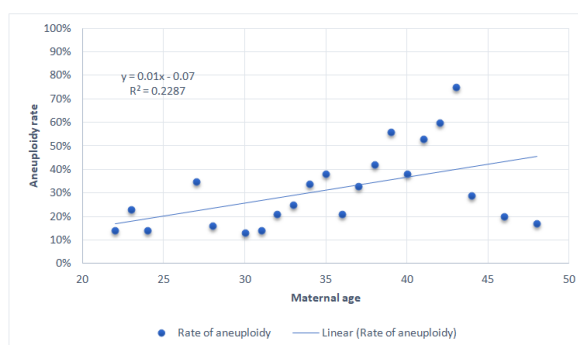Abnormalities probity of chromosomes = -0.7 + 0.01 x (maternal age)

Figure 3: The linear regression line shows the correlation between maternal age and the rate of aneuploidy. R^2: coefficient index

Discussion

The evaluation of genetic normality embryo is the most important criterion for the selection of embryos to be transferred. Thus, the present study attempted to re-evaluate previous studies that had described the relationship between chromosome abnormalities and maternal age using newest PGS method based on NGS technology [10], [11], [12], [13], [14], [15]. The analysis of more than 603 embryos for 23 chromosome pairs makes this study the most extensive and comprehensive to date on chromosome abnormalities in human cleavage stage embryos.

In this study showed that the abnormal chromosomal IVF in Vietnam population was 42.7% (Confident interval 95%). This number indicated with Rubio's study in 2003 analyzing similar maternal age that there were 45.1% embryo had abnormal chromosomal [16]. In 2003, Alfarawati showed that there was more than half of embryos tested had abnormal (56.7%) [17]. However, their study focused on recurrent miscarriage and high maternal age (average 37.5 years) whereas this study focused on patient had average age lower (34.4 years). In 1991, Zenzes and Casper represented the rate of abnormalities in embryo was fluctuate from 23% to 40% [18]. It is possible to project used NGS method with high accuracy compare with karyotyping used by Zenzes.

Aneuploidy is the most error observed on the embryo at the blastocyst stage [19]. Notably, the proportion of each chromosome is the difference in order that testing individual chromosomal or some chromosomes was concluded healthy embryo due to an increasing false negative. Therefore, the high aneuploidy rates observed suggest that chromosome screening at the blastocyst stage may be beneficial, particularly for women >34 years [20].

On the other hand, the results agreed with previous trends, such as that aneuploidy increases in cleavage stage embryos with maternal age. It is clear that maternal age is only one of the causes of chromosome abnormalities. The present study confirms that only 61.95% of embryos from young patients are healthy for the chromosomes studied (< 37 years), and this frequency decreases to 45.83% with advancing maternal age (>37 years) (CI: 95%, p < 0.05). In 2003, Rubio's study illustrated that the abnormalities rate of chromosomes were 33.3% at patients age <37 years and increased to 57.7% at patients age >37 years [16]. Of research made, Aneuploidy reached over 50% with maternal age >40 years [20]. In addition, in the year 2017, Upadhyaya concluded that the number of normal embryos in group < 37 years was 65.5% and fallen at 40.6% when maternal age increasing [21]. This proportion for this study was very similar to the proportion of our study. Even Upadhyaya et al. used another

technology (array CGH) to detected comprehensive chromosomes.

The results make us believe that chromosomal abnormalities of the embryo in IVF blastocyst stage have a relationship with maternal age, although the correlation coefficient is not really high (r = 0.4783, CI:95%). In fact, this coefficient is positive, that means the higher the mother's age will have more chromosomal abnormalities of embryos. Figure 3 shows that each 22.87% variation in the incidence of chromosome abnormalities is explained by the variation of maternal age factor (coefficient of determination R-square = 0.2287). When maternal age increases by one unit, the incidence of chromosome numbers increases by 0.01 units, and this change is significant, with 95% confidence [22].

Overall, the probability of embryo aneuploidy increases when maternal age higher. This effect becomes more impressive for some chromosomes. The chromosome most affected by age is chromosome 14; the data show that the rate of multiple deviations increased by 3.7 times in the elderly group (≥ 37 years). Then came chromosome 7, the rate of multiple deviations increased by 3.3 times and chromosome 18 increased by 2.9 times when the mother's age increased compared with the average increase of about 1.5 to 2 times for all chromosomes 2, 3, 12, 13, 15, 17, 18, 19, 20, 21, 22. In addition to the study of Alfarawati and colleagues in 2011, chromosomes 7 and 14 were the most prevalent group with the highest increase in maternal age (5-6 times higher), and for infection chromosomes 2, 15, 17, 20, 22 doubled [17]. This conclusion is quite similar to our results, thus confirming the reliability of this study.

In conclusion, the rate of chromosomal abnormalities of embryos at blastocyst stage in vitro fertilization in the Vietnamese population is 42.7%. The most common groups of chromosomes were observed including chromosome number 15, 16, 21, 22 and sex chromosomes.

The proportion of embryos with abnormal chromosome and maternal age has a positive correlation with the average level of contact (r = 0.4783), meaning that the higher the age of the mother will make greater the risk of creating embryos have chromosomal abnormalities.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable

ethical standards. This study was approved by the ethics committee Vietnam Military Medical University.

Informed consent

The informed consents were signed by patients.

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