Next generation sequencing for preimplantation genetic testing of blastocysts aneuploidies in women of different ages

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Abstract
Most of the current preimplantation genetic screening of aneuploidies tests are based on the low quality and low density comparative genomic hybridization arrays. The results are based on fewer than 2,700 probes. Our main outcome was the association of aneuploidy rates and the women’s age. Between August–December 2013, 198 blastocysts from women (mean age 36.3±4.6) undergoing in vitro fertilization underwent routine trophectoderm biopsy. NGS was performed on Ion Torrent PGM (Life Technologies). The results were analyzed in five age groups (<31, 31–35, 36–38, 39–40 and >40). 85 blastocysts were normal according to NGS results. The results in the investigated groups were (% of normal blastocyst in each group): <31 (41.9%), 31–35 (47.6%), 36–38 (47.8%), 39–40 (37.7%) and >40 (38.5%). Our study suggests that NGS PGD is applicable for routine preimplantation genetic testing. It allows also for easy customization of the procedure for each individual patient making personalized diagnostics a reality.

Key words
Preimplantation genetic diagnosis (PGD), next generation sequencing (NGS), blastocyst

INTRODUCTION
It is common knowledge that a high incidence of chromosome aneuploidy in human oocytes and embryos contributes to low implantation and pregnancy rates. Nowadays, it is known as the most frequent and important cause of low human fecundity. These aneuploidies mostly occur due to chromosome segregation errors during female meiosis and less often during consecutive embryo mitosis. Male meiosis is a rare cause of embryonic aneuploidies. It is crucial to solve this problem. Testing embryos prior to transfer could increase the pregnancy rate, decrease miscarriages and prevent multiple IVF cycles in a patient with a very high incidence of aneuploidy, and consequently a low or zero chance of achieving a pregnancy with her own oocytes [1, 2, 3].

The first of these technologies, still in use today, is microscopic morphology analysis. However, there are numerous developmental abnormalities which do not affect embryo morphology [4, 5], and even at the blastocyst stage almost 50% of good morphology embryos can be aneuploid. A step forward was the introduction of the time-lapse system for continuous morphokinetics analysis. Although many results show progress in results obtained, it is accepted not because of the data accuracy but due to the noninvasiveness of the method. For that reason, more reliable, but invasive, diagnostic tests are in use; the results, however, are more accurate and misdiagnosing occurs much less often. Preimplantation genetic screening/diagnosis (PGS/PGD) is the fastest growing part of IVF treatment in developed countries.

The first of the widely used methods, fluorescence in situ hybridization (FISH), is based on fluorescent probes connected to known parts of chromosomes sequences. It is used to find selected chromosomes and estimate their number, even in the interphase nuclei. This method is easy to perform but the results are difficult to interpret and ambiguous, and could also be dependent on the phase of the cell cycle of the investigated cells. Although it is not possible to check more than 5 chromosomes in parallel, it is possible to perform rehybridisation of the sample, but only up to three times due to the destruction of the investigated DNA.

Most of the current preimplantation genetic screening tests for aneuploidies are based on the low quality and low density comparative genomic hybridization arrays, the results of which are based on fewer than 2,700 probes. This means that it is rather a multiple FISH method than an actual array. New technical possibilities, such as next generation sequencing technique, allow improvement of the the diagnostics [6, 7]. The complication of the method decreases accessibility and pushes the diagnostics into specialized, central laboratories. Preimplantation genetic diagnosis has become one of the most important methods for prioritization of embryos for transfer. Most of the selection methods used previously were based on the detailed morphology of embryos. It was shown that the optimal morphological characteristics can be correlated with higher implantation rates; however, morphology is only a weak predictor of implantation rate and ploidy [4, 8,
9, 10. In the research by Yang, the clinical pregnancy rate was significantly higher in the morphology+aCGH group, compared to the morphology-only group (70.9 and 45.8%, respectively \( p=0.017 \)); ongoing pregnancy rates for the same were 69.1 vs. 41.7%, respectively \( p=0.009 \) [11].

OBJECTIVES

The aim of the presented study was to check the influence of women's age on aneuploidy rates, and to evaluate the frequency of aneuploidies among women in different age groups to find the cut-off age for PGD NGS procedures.

MATERIALS AND METHOD

IVF treatment cycles were performed in accordance with the authors' standard GnRH agonist long protocol, using 225 IU of human menotropin (Menopur, Ferring Pharmaceuticals) for about 9 days of controlled ovarian stimulation [12]. There were different reasons for IVF procedures – advanced maternal age, recurrent pregnancy loss, recurrent implantation failure. From August to December 2013, 198 blastocysts from women (mean age: 36.3±4.6) undergoing in vitro fertilization with preimplantation genetic testing were biopsied for next generation sequencing. All of the embryos were vitrified for FET (frozen embryo transfer), which was 2.8 embryo per woman, on average. Aspirated trophectoderm cells from blastocysts were transferred into thin-walled 0.2mL PCR tubes in total volume of 2.5 ul and subjected to lysis. Whole genome amplification (WGA) was performed using the Rubicon GE test. The concentration of DNA after WGA was quantified with Qubit 2.0 Fluorometer and Qubit dsDNA HS Assay. Ion Xpress Plus gDNA Fragment Library Kit (Life Technologies) was used for library preparation in accordance with manufacturer's protocol. Libraries were cloned and amplified with the Ion PGM™ Template OT2 200 Kit (Life Technologies) using the Ion One Touch 2 System. After chip loading, sequencing was performed using Ion PGM™ Sequencing 200 Kit v2 (Life Technologies) on Ion 314 and 316 chips. Preliminary analysis, e.g. base calling and read mapping against human genome reference sequence (Hg19) were performed with Ion Torrent Suite Software. The Invicta Bioinformatics Team Script was used for further computational calculations. Read coverage for each chromosome was corrected for GC-bias, and aneuploidy detection was performed using sample results comparison to baseline values obtained from 72 male and 52 female samples processed beforehand with the established protocol, as described above. The Invicta algorithm was introduced to eliminate the influence of sample-to-sample reads coverage variance on false-positive calls. Male control samples were processed together with trophectoderm cells from blastocysts, and underwent the same computational analysis to exclude any performance malfunctions, and the negative control sample processed to exclude contaminations.

The protocol was prepared for low resolution in order to obtain 50,000 amplicons per blastocyst, specifically for aneuploidies screening. The exact types of aneuploidies in different women's age groups were also examined and the undiagnostic result rates calculated. The results were analyzed in five age groups (<31, 31–35, 36–38, 39–40 and >40).

RESULTS

85 blastocysts were normal according to NGS results. Table 1 shows the number of examined and healthy blastocysts in each age group. It was found that the average percentage of blastocysts affected by aneuploidies was 56.6% and varied between 52.2–62.3% in different age groups. No nondiagnostic results were obtained.

Table 1. Prevalence of euploid blastocysts in different age groups

<table>
<thead>
<tr>
<th>Age group (years)</th>
<th>No. of blastocysts</th>
<th>No. of normal</th>
<th>% of normal in age group</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;31</td>
<td>31</td>
<td>13</td>
<td>41.9%</td>
</tr>
<tr>
<td>31–35</td>
<td>42</td>
<td>20</td>
<td>47.6%</td>
</tr>
<tr>
<td>36–38</td>
<td>46</td>
<td>22</td>
<td>47.8%</td>
</tr>
<tr>
<td>39–40</td>
<td>53</td>
<td>20</td>
<td>37.7%</td>
</tr>
<tr>
<td>&gt;40</td>
<td>26</td>
<td>10</td>
<td>38.5%</td>
</tr>
</tbody>
</table>

Table 2 shows the kind of aneuploidies that were found in the different age groups. More than half were the disturbance of single chromosome, but almost 24% were complex ones.

Table 2. Types of aneuploidies found in different age groups

<table>
<thead>
<tr>
<th>Age group (years)</th>
<th>1M</th>
<th>1T</th>
<th>1M and 1T</th>
<th>2M</th>
<th>2 T</th>
<th>2 M and 2 T</th>
<th>Complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;31</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>31–35</td>
<td>9</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>36–38</td>
<td>4</td>
<td>6</td>
<td>7</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>39–40</td>
<td>11</td>
<td>9</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>&gt;40</td>
<td>4</td>
<td>3</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Total n</td>
<td>33</td>
<td>25</td>
<td>16</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>27</td>
</tr>
</tbody>
</table>

M – monosomy; T – trisomy

Table 3 shows the prevalence of different chromosomes aneuploidies. Only aneuploidies of chromosome 22 were much more frequent than any other. There was also a trend for higher aneuploidies prevalence among small chromosomes.

Table 3. Aneuploidy prevalence for different chromosomes

<table>
<thead>
<tr>
<th>Prevalence %</th>
<th>0–2%</th>
<th>2–4%</th>
<th>4–6%</th>
<th>6–8%</th>
<th>8–10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>chromosome</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,6,8,10,12,14</td>
<td>7,9,15,17</td>
<td>2,3,4,11,16,18</td>
<td>5,13,19,20,21</td>
<td>X,22</td>
<td>15.6%</td>
</tr>
</tbody>
</table>

Figures 1 and 2 present examples of results for normal and aneuploid embryos. The differences of abnormal signals from chromosomes are unambiguous and easy to interpret.

DISCUSSION

The gold standard in the treatment of infertility would be to achieve a single cell fertilization, which would create a genetically normal embryo providing a single pregnancy after only one transfer.
To date, no way has been found to influence the genetic status of oocytes. Human reproduction is based on cells of which only about 25%–50% are genetically normal; therefore, the treatment has to be based on the diagnosis of the derived cells – embryos. Providing optimal diagnosis, the chances of implantation could be increased and a significant reduction achieved in the number of miscarriages. This is of particular importance in the treatment of women over 35. In such cases, the time factor is also of major importance.

On one hand, such situations deal with a decrease in the ovarian reserve, which significantly affects the reduction in the chances of pregnancy [13]; on the other hand, a significant increase in aneuploidy risk limits the possibility of finding a healthy embryo.

In the case of resignation from preimplantation genetic diagnosis, in most cases pregnancies can be obtained, however in more than 20% ending in a miscarriage. Such an algorithm of treatment reduces the chances of giving birth to a healthy baby. Patients after miscarriages may be recovering for even 6 months to the state of health that allows them to attempt the next IVF cycle – a delay which they cannot afford.

On the other hand, attention should be paid to the detection of aneuploidy in young women’s blastocysts. Typically, preimplantation diagnosis is not recommended for young women. Some of the examined blastocysts were from young women with low ovarian reserve (extremely low AMH <0.4 ng/ml). These are women with poor prognosis of pregnancy [14].

So far, poor pregnancy prognosis relying on a low ovarian reserve has meant a small supply of cells, and as a result, smaller amounts of derived embryos. Contrary to expectations, it was found that as many as 58.1% of blastocysts obtained from these women exhibited genetic abnormalities. This raises the suspicion that genetic disorders are not the only reason of major decline in implantation rates in older women. Only when considering reduced ovarian reserve affecting the increase in aneuploidy rate, the low performance in assisted reproduction centres can be explained.

A significant increase in the aneuploidy rate was observed from the age of 39, which confirms previous observations of a cut-off age when the effectiveness of the treatment after a woman reaches the age of 38 [17].

The presented study also examined the types of abnormalities identified during analysis using next generation sequencing. The occurrence of a single monosomy was found in about 29% of cases, a single trisomy in 22%, and other disorders such as double monosomy and trisomy in almost 25% of cases. Yang et al. [11] showed the analogous results using aCGH: 36%, 21% and 29% (respectively). Yang’s results strongly differed in the amount of complex aneuploidies – 14.7% vs. 23.9% in the current study. However, after the exclusion of complex disorders (considered to be of mitotic origin), the rates of single and dual abnormalities in the current study were 67.4–66.3%, compared to the results obtained by Yang for individuals 32.5–33.7%, respectively. The differences in the detection of complex chromosome abnormalities could be a result of the greater sensitivity of next generation sequencing in comparison with aCGH.

In the presented study, only aneuploidies connected with chromosome 22 showed a prevalence rate much higher than for other chromosomes. The remaining aneuploidies appear to be rather accidental errors without any regularity. Verlinsky et al. [15] showed the highest frequency of aneuploidies in small chromosomes, especially 21 and 22. Similar findings are described by Jóźwiak et al. using MLPA reaction [16].

In the presented study, low resolution sequencing was used as it is sufficient for detecting quantitative abnormalities of chromosomes. The average number of reads was 55,000, which is about 20 times more than the coverage of routinely-used arrays. By using NGS, the resolution could easily be adjusted to optimize the cost of obtaining results, which is not possible in the case of applying the aCGH.

CONCLUSIONS

A larger study is needed to exclude possible biases connected with the different causes of patient infertility. Cross-laboratory validation is also necessary and will be possible when other PGD laboratories switch from aCGH to NGS technology.

The presented study suggests that NGS PGD is applicable for routine genetic preimplantation testing. It provides much more data than aCGH and allows adjustment of the diagnostics for each patient individually. Personalized diagnostic tools are probably only a small step towards achieving a goal of holistic, personalized treatment for each patient.

REFERENCES