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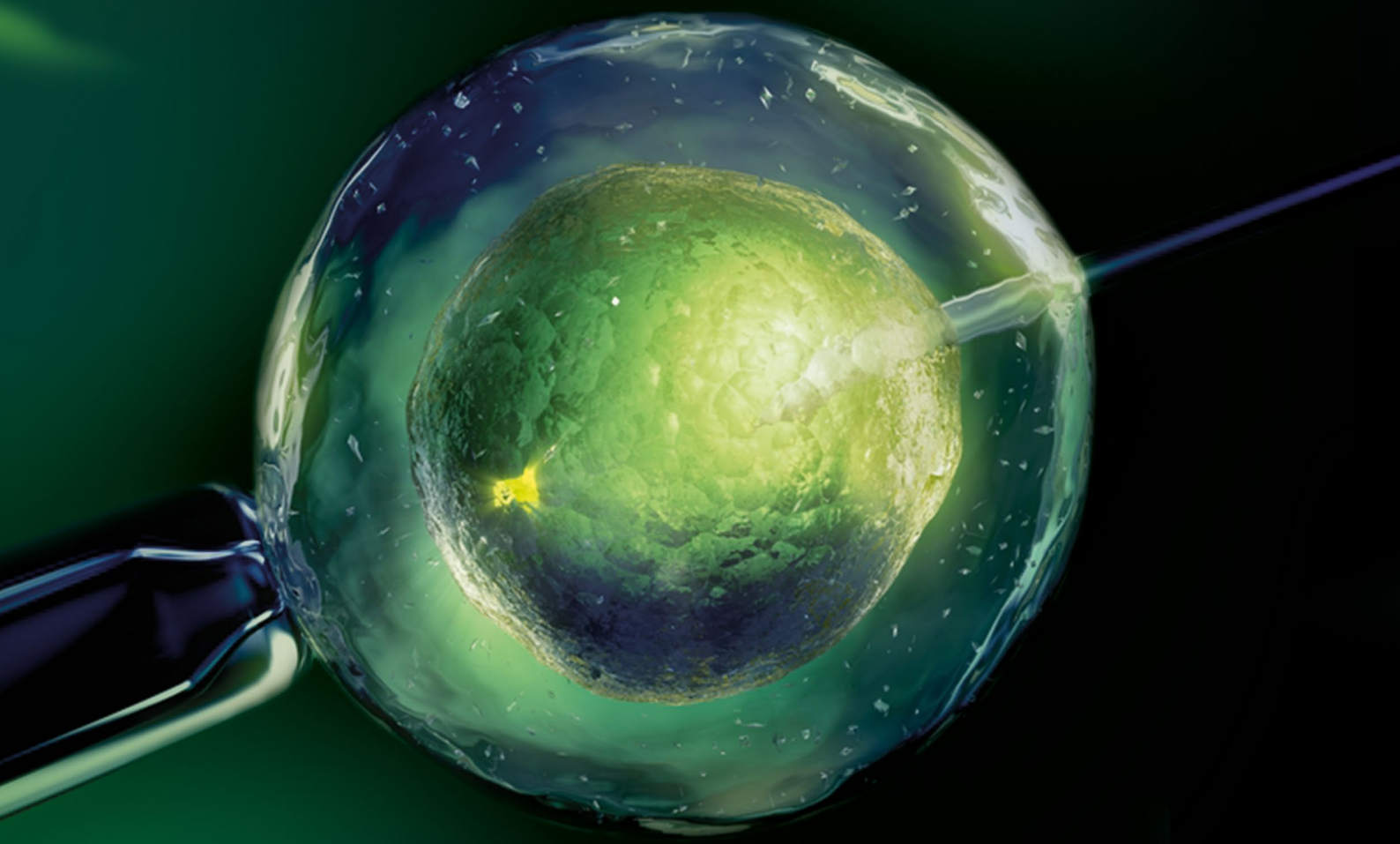

The impact of next generation sequencing (NGS) preimplantation genetic diagnostics (PGD) on pregnancy rate of frozen blastocyst embryo transfer.

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Routine use of next generation sequencing (NGS) for preimplantation genetic diagnostics (PGD) of blastomeres obtained from embryos on day 3 in fresh IVF cycles – a prospective case control study.

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Routine use of next generation sequencing (NGS) for preimplantation genetic diagnostics (PGD) of blastomeres obtained from embryos on day 3 in fresh IVF cycles – a prospective case control study.

Abstract

Objective:
To determine the usefulness of semiconductor-based next generation sequencing (NGS) for cleavage-stage preimplantation genetic diagnosis (PGD) of aneuploidy.
Design:
Prospective case control study.
Setting:
A private centers for reproductive medicine.
Patient(s):
28 patients underwent day-3 embryo biopsy with PGD and fresh cycle transfer. Additionaly, 106 patients, matched according to age, anti-müllerian hormone (AMH) levels, antral follicle count (AFC) and infertility duration were selected to serve as controls.
Intervention(s): Choice of the embryos for transfer was based on the PGD NGS results.
Main Outcome Measure(s):
Clinical pregnancy rate (PR) per embryo transfer was our primary outcome. Secondary outcomes were the implantation rate, and miscarriage rate.
Result(s): The pregnancy rate was higher in the NGS group (77.8% vs. 45.3%, p<0.001). The implantation rate (48.2% vs. 33.9%, p<0.3) was higher in NGS group. The miscarriage rate was lower in NGS group (4.8% vs. 6.3 %, p<0.8).
Conclusion(s):
We demonstrate the technical feasibility of NGS-based PGD involving cleavage stage biopsy and fresh embryo transfers. Encouraging data was obtained from a prospective trial using this approach, arguing that cleavage stage NGS may represent a valuable new addition to the aneuploidy screening methods currently available. Although encouraging, this findings require further validation in a well-designed randomized controlled trial.

Introduction

Only 30% of the natural conceptions have been estimated to result in a successful pregnancy [1], [2]. Similarly, the majority of in vitro fertilization (IVF) cycles fail to produce a child. In order to maximize the likelihood of successful infertility treatment, the embryos produced undergo a morphological evaluation, the aim being to identify and transfer the embryo(s) with the greatest potential for forming a viable pregnancy. Unfortunately, standard methods of morphological assessment are only weakly predictive of implantation rate [3], [4].
It is well established that aneuploidy is

extremely common in human oocytes and that such abnormalities are a leading cause of miscarriages and congenital birth defects [5], [6]. Additionally, aneuploidy has a significant impact on implantation and pregnancy rate, since many chromosome abnormality are also observed in material from younger women. For example, at the cleavage stage aneuploidy rates have been reported varying embryos, vary from 50% in young women in age under 35 to even more than 90% in those over the age of 42 [7], [8] [9]. The high frequency of aneuploidy during preimplantation development and the likely deleterious effects on embryo viability, have led to the suggestion that embryos should be tested for chromosome abnormalities prior to deciding which to transfer to the uterus [10]. However, routine morphological analysis reveals very little concerning the chromosomal status of an embryo [10]. For this reason, preimplantation genetic screening (PGS) has been developed. Techniques for PGS involve the sampling (biopsy) of polar bodies, single blastomeres, or trophectoderm cells, followed by cytogenetic analysis to distinguish chromosomally normal embryos from those affected by aneuploidy. Embryos found to be chromosomally normal can then be prioritized for transfer. Despite earlier controversies concerning the efficiency of PGS, recent data using comprehensive chromosome screening methods has been associated with encouraging data, including improved implantation and pregnancy rates in randomized controlled trials [11], [12], [13], [14]. Although, PGD for aneuploidy is emerging as a powerful tool for embryo selection, even more efficient methods are still required. Ideally new approaches should give reliable information on chromosome copy number at lower cost than existing methods and provide to possibility of more detailed genetic information, improving patient access to aneuploidy screening and shedding more light on embryo viability. The introduction of next generation sequencing into routine clinical use, as decribed in this paper, represents an important step towards these goals.

Materials and Methods

A prospective study was performed at a private fertility clinic. The study population consisted of patients treated by intracytoplasmic sperm injection (ICSI) between 08.2013 and 01.2014. 28 patients decided to undergo preimplantation genetic diagnosis of aneuploidy because of repeated implantation failures. Each has previously undergo at least 2 unsuccessful IVF cycles. A control group was created consisting of 106 patients, matched to the PGD patients in term of infertile etiology, number of failed cycles, age and range of hormonal and other prognostic markers (Table 1). Controls and PGD patients underwent treatment during the same time interval. Patient selection was facilitated using our database and medical software (Invictus ver. 3.1.62 Invicta Limited.)

Stimulation Protocol

All women were treated on a long agonist protocol starting from oral contraceptive (OC) pills (Ovulastan, Adamed, Czosnow, Poland) from 2nd-5th day of the cycle. Triptorelin acetate 0.1 mg (Gonapeptyl, Ferring, Saint-Prex, Switzerland) was administered 14 days after the beginning of OC.

Fourteen days later (7 days after the end of OC) the administration of urinary gonadotropins (Menopur, Ferring, Saint-Prex, Switzerland) for ovarian stimulation was initiated, the dosage, depending on the anti-müllerian hormone (AMH) level (from 150 to 300 IU daily) [15].

Monitoring of follicular growth was carried out on day-8 by ultrasonographic scan and assays of serum estradiol (E2), progesterone (P) and luteinizing hormone (LH). Oocyte pick-up was performed 36 hours after the administration of 5,000 IU of human chorionic gonadotropin hCG (Choragon, Ferring, Saint-Prex, Switzerland).

The luteal phase was supplemented by transvaginal progesterone (3x100 mg Lutinus, Ferring, Saint-Prex, Switzerland and estradiol (3x2 mg, Estrofem NovoNordisk, Bagsvaerd, Denmark) and hormone levels (E2, P and hCG) were checked every 3-4 days.

Preimplantation Genetic Diagnosis

Single blastomere biopsy of cleavage stage embryos composed of 6-8 cells was performed on day-3 of culture. Embryos underwent biopsy individually in Ca2+ - free biopsy medium (Vitrolife, Vastra Frolunda, Sweden). The criteria for selection of blastomeres were the presence of the nucleus and maintenance of cell integrity.

The zona pellucid was breached using a 1488 nm 300 mW Anritsu laser incorporated into Saturn 3 system (Research Instruments, Falmouth, UK).

After biopsy the embryos were washed and transferred to blastocyst growing medium (Vitrolife, Vastra Frolunda, Sweden). The collected blastomeres were washed in phosphate-buffered saline (PBS)/polyvinyl-pyrolidone (PVP), then transferred to 0.2ml PCR tubes in a total volume of 5 µl. Blastomeres were subjected to lysis and whole genome amplification (WGA) prior to construction of sequencing libraries. Whole genome amplificated DNA concentration was quantified with Qubit 2.0 Fluorometer and Qubit dsDNA HS Assay, and Ion Xpress Plus gDNA Fragment Library Kit (Life Technologies, California, USA) was used for library preparation according to manufacturer's protocol. Barcoded libraries were clonally amplified with The Ion PGM™ Template OT2 200 Kit (Life technologies, California USA) using Ion One Touch 2 System. After chip loading the sequencing was performed using Ion PGM™ Sequencing 200 Kit v2 (Life technologies, California USA) on Ion 316 chips. Preliminary analysis e.g. basecalling and reads mapping against human genome reference sequence (Hg19) was 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 85 male and 83 female samples processed beforehand with the same method. Positive (normal male) and negative controls were processed to confirm appropriate functioning of the test and exclude contaminations.

Statistical Analysis

The statistical package StatSoft, Inc. (2011) STATISTICA (data analysis software system), version 10 (www.statsoft.com) was used for data analysis. The clinical characteristics in the investigated group were compared using Mann Whitney U test. The Pearson Chi-square and Fisher test were used to assess differences among groups with regard to different rates of development. A value of $P<0.05$ was considered as statistically significant.

Ethics Statement

Institutional Review Board

We obtained written consent from each couple. All data were de-identified and analyzed anonymously. Moreover, we obtained informed consent to present our data in any publication as long as confidentiality was maintained. These conditions were met and the study was approved by the University of Varmia and Masuria, Olsztyn, Poland Institutional Review Board.

Results

During the study 28 patients underwent PGD for aneuploidy using NGS. The control was composed of 106 patients selected from patients who were undergoing treatment in our clinic at the same time. All patients suffered a history of repeated implantation failures (≥2). The conrol group was matched according to age, cause of infertility, duration of infertility, AMH level and antral follicles count. They did not differ in other hormonal parameters which we routinely verify (Table 1).
Finally we performed 28 cycles in the investigated group. The average number of retrieved oocytes was relatively low (8.4 oocytes per patient) but sufficient to find at least one chromosomally normal embryo in 27 cases (96.4%). The pregnancy rate (as ultrasound fetal heartbeat detection on 6 weeks and 1-3 days) per transfer was almost two times higher in the group having NGS-based embryo selection compared to control group. An increase was also found in implantation rate (Table 2).

Table 1. Characteristics of the treatment group.

Variable	PGD NGS group	Control group – no PGD	p value
No. of subjects	28	106	
Mean (SD) age	34.7 (3.8) 35 (31-38)	35.3 (2.1) 35 (34-37)	0.27
BMI (kg/m2)	22.1 (3.1)	22.4 (3.6)	0.42
Mean (SD) duration of infertility (years)	4.2 (2.8)	4.3(2.9)	0.82
Mean (SD) IVF cycles done before	2.58 (0.97)	2.40 (0.74)	0.38
AMH (ng/ml)	2.6 (1.7) 2.2 (1.3-3.5)	2.6 (0.6) 2.6 (2.02-3.1)	0.27
Inhibin B	55.6 (44.5)	65.2 (36.7)	0.29
basal FSH b	7.4 (7.5)	7.6 (7.4)	0.52
basal LH (SD)	7.2 (6.08)	7.1 (5.38)	0.85
basal E2	46.1 (31.6)	47.3 (31.5)	0.67
DHEAS	203.6 (94.2)	201.5 (77.8)	0.53
Testosterone	1.4 (1.3)	1.6 (1.8)	0.12
SHBG	74.2 (36.4)	68.7 (38.1)	0.23
AFC	15.1 (8.1) 13 (10-20.5)	15.7 (3.5) 15 (13-18)	0.12

a body mass index; b follicle-stimulating hormone; c dehydroepiandrosteron-sulfate; d sex hormone-binding globulin

Table 2. In vitro fertilization program characteristics of the investigated groups.

Variable	PGD NGS group	Control group – no PGD	p value
No. of cycles	28	106	
No. of transfers	27	106	
Duration of stimulation– days(SD)	8.2 (2.1) 8 (8-9)	8.1 (0.8) 8 (7-9.5)	0.54
hMG dose (IU)	2032.5	2167.5	0.14
No. oocytes retrieved (SD)	8.4 (4.1) 8 (7-9.5)	8.4 (3.9) 7.5 (6-11)	0.63
Fertilization rate (%)	74.6	67.3	0.16
Number of embryos biopsied (mean per cycle)	145 (5.2)	-	
NGS results (% of biopsied embryos):			
Normal	48 (33.1)	-	
Abnormal	84 (57.9)	-	
No diagnosis	13 (8.9)	-	
Read number (SD) per embryo	61372.2 (9026.0)	-	
Average No. of embryos transferred	1.3	1.6	0.003
Pregnancy rate (per ET - %)	21 (77.8)	48 (45.3)	0.001
Implantation rate (%)	48.2	33.9	0.3
Multiple pregnancy rate of pregnancies (%)	1 (4.8)	16 (33.3)	0.001
Ectopic pregnancy (%)	1 (4.8)	0	0.13
OHSS (%)	0	0	
Biochemical pregnancy	0	4 (8.3)	0.17
Spontaneous abortion below 12 weeks rate (%)	1 (4.8)	3 (6.3)	0.8

hMG - human menopausal gonadotropin; ET – emryotransfer; OHSS - ovarian hyperstimulation syndrome

Discussion

Aneuploidy is seen in more than half of cleavage-stage embryos derived from women in their early thirties and becomes still more common with advancing age, exceeding 90% of embryos affected for patients over 42 years of age [9]. Since the vast majority of aneuploidies are lethal, it is been argued that IVF outcomes could be improved if embryos are screened for aneuploidy, with those found to be chromosomally normal given priority for transfer to the uterus[10]. This concept is sometimes referred to as preimplantation genetic screening (PGS).

Despite earlier controversy concerning efficiency, the results of recent randomized clinical trials has suggested that PGS may indeed be beneficial, improving selection of viable embryos and thereby increasing implantation rate and reducing the incidence of miscarriage. It is increasingly common for PGS to be used in conjunction with biopsy at the blastocyst stage, followed by cryopreservation and transfer of those found to be euploid in a subsequent cycle. While this strategy generally works well, the cryopreservation of embryos represents an additional complication, adding to the costs of the procedure and causing logistic challenges in terms of laboratory patient management. Additionally, many patients have a strong preference for fresh embryo transfer. For this reason, techniques that allow reliable detection of aneuploidy within a timeframe compatible with a fresh embryo transfer continue to have an important place in clinical practice.

The study described in this paper examined the feasibility of utilizing emerging NGS methods for the detection of aneuploidy in single blastomeres biopsied at the cleavage stage. Utilization of NGS has several potential advantages over existing comprehensive chromosome screening methods, including reduced costs and the potential to provide additional genetic (DNA sequence) information, which may be of clinical relevance. Our studies have previously shown that NGS can provide accurate cytogenetic analysis of single cells [16], [17]. However, few have reported clinical application and there has only been one previous report of a methodology sufficiently fast to allow testing using NGS and transfer within the same cycle [18]. We confirmed that a rapid NGS methodology, providing results within 12 hours, can be successfully utilized in a routine clinical scenario, permitting fresh embryo transfers.

A randomized clinical trial using the NGS technology described in this paper is yet to be performed and is clearly needed in order to make firm conclusions concerning the value of the process. However, the prospective study undertaken has yielded some interesting and encouraging clinical data. Hereby, in order to minimize the costs of NGS, it was usual to analyze cells from on average 5.2 embryos simultaneously. On average more than 60 000 reads were obtained per sample. This represents 10 times more data points than obtained using widely used embryo testing methods based upon aCGH (e.g. Blue Gnome [2684x2]), increasing statistical confidence in the results delivered. The average percentage of chromosomally normal cleavage stage embryos was 33.1%. Although NGS can be used for sequencing of entire genomes, we did not attempt to achieve whole genome coverage. For this purpose of aneuploidy detection, relatively few fragments of DNA sequence ('reads') are required from each chromosome. We opted to concentrate on loss or gain of entire chromosomes and not to look for subchromosomal abnormalities (e.g. segmental aneuploidy). The use of NGS data for high-resolution examination of chromosomes inevitably means a reduction in the number of reads upon which the diagnosis is based and may consequently reduce accuracy. Furthermore, the clinical relevance of segmental aneuploidy at the cleavage is unclear at this time. Embryonic cells do not transition trough G1, G2 or G0 phases of the cell cycle, but rather move directly from mitosis to synthesis phase [19]. This fact, coupled with asynchrony between replication origins can cause false positive aneuploidies in fluorescence in situ hybridization (FISH), aCGH and PCR based methods. The low resolution sequencing described in this paper diminishes the influence of variation in replication across the genome and is therefore less susceptible to the detection of spurious segmental abnormalities. This assertion is indirectly confirmed by our results. Despite the fact that NGS assess the copy number of all 24 types of chromosome, the aneuploidy rate was actually lower than seen in equivalent FISH studies (which only assessed up to 9 chromosomes). The high pregnancy rate and lower miscarriage rate observed in the clinical NGS cycles also suggest that few if any errors were made using the reported protocol.

As expected, the number of embryos available for transfer following NGS was much lower in comparison to cycles without PGS. Our policy was to transfer 2 embryos per patient where possible. However, we had only 1.3 embryos eligible for transfer on average in NGS group. The implantation rates achieved were considered higher than typically observed in our clinic and higher than the contemporary control group assembled for the purpose of this study. The identification of embryos with high implantation potential and exclusion of those affected by lethal aneuploidies allowed us to reduce the number of embryos cryopreserved (or in many cases eliminate cryopreservation altogether), thus reducing patient costs and circumventing ethical issues associated with the prolonged storage of embryos.

Pregnancy and implantation rates achieved in cycles utilizing NGS exceeded expectation and suggest that this approach is improving identification of viable embryos. However, the relatively small size of the investigated group and lack of randomization are acknowledged weaknesses of this investigation. While we will be continuing our research, we strongly encourage other centers to initiate studies that can provide additional, more robust data, revealing the true efficacy of NGS-based preimplantation aneuploidy testing at the cleavage stage.

The investigated group did not include patients who suffered from endometriosis or adenomiosis. We also excluded patients with problems related to

endometrium thickness. These groups will probably remain a challenge for effective treatment. There is a need for the more investigations into the efficacy of PGS applied to these type of patients.

In the current study, only one miscarriage occurred in the group that received screening using NGS, whereas in control group without embryo genetic testing three miscarriages occurred before 12 week of pregnancy and additional four after biochemical pregnancy was stated . Although the sample size was insufficient to demonstrate whether or not this represents a significant reduction in miscarriage risk, the result suggest that embryo selection using NGS technology possess strong potential to decrease such risk. Further investigations including more patients should be applied due to confirmation.

Conclusion

This paper confirms that NGS can be applied clinically for the purpose of detecting aneuploidy in human preimplantation embryos. Furthermore, we demonstrate that NGS is not only applicable to trophectoderm biopsies, but also to single cells and that the method described, based upon the use of semi-conductor sequencing , is sufficiently rapid to allow NGS in combination with a fresh embryo transfer. Data from a prospective trial indicated that single blastomere biopsy followed by fresh transfer of euploid embryos on day-5 was associated with excellent implantation and pregnancy rates and a low miscarriage rate, suggesting that NGS may be a useful tool for embryo selection. Our results require confirmation in different clinics, preferably in the context of a randomized clinical trial, but if confirmed there will be a strong argument for the wider application of genetic methods for the ascertainment of embryo viability.

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