J Assist Reprod Genet (2016) 33:1467–1471
DOI 10.1007/s10815-016-0766-5

GENETICS

Reanalysis of human blastocysts with different molecular genetic screening platforms reveals significant discordance in ploidy status

Drew V. Tortoriello[1] · Molina Dayal[1] · Zeki Beyhan[1] · Tahsin Yakut[2] · Levent Keskintepe[1,2]

Received: 30 March 2016 / Accepted: 30 June 2016 / Published online: 16 July 2016

Abstract
Objective The objective of this study is to determine mosaicism and its effect on blastocysts; abnormal blastocysts determined by molecular testing were sequentially biopsied and retested.
Material and method We re-biopsied 37 blastocyst-stage abnormal embryos from eight patients, which were reanalyzed to determine the level of concordance between biopsies and inter-laboratory congruence between reputable commercial PGS laboratories.
Results The main outcome measures were intra-embryo variation between sequential embryo biopsies and inter-laboratory variation between two PGS laboratories. The compatibility between both aCGH and NGS was found to be 11 % (3/27). Importantly, 9/27 (33 %) of embryos originally reported to be aneuploid, upon repeat assessment, were found to be euploid. The concurrence for SNP array and NGS was 50 % (3/6), and 17 % (1/6) of these abnormal embryos tested normal upon re-evaluation with NGS. NGS resulted 41 % (11/27) normal results when 27 of CGH abnormal embryos were retested. Concordance between aCGH and NGS was 4 % (1/27) whereas in three instances, gender discrepancy was observed with NGS when aCGH abnormal embryos were reanalyzed.
Conclusions The results of these studies reinforce the prevalence of inconsistencies during PGS evaluation of trophectoderm biopsies possibly due to variations in platform sensitivity and heightening concerns over the clinical tractability of such technology in human ARTs..

PGS · Embryo biopsy · Mosaicism · Aneuploidy

Capsule Blastocyst mosaicism were evaluated for the consistency of preimplantation genetic screening procedures.

✉ Levent Keskintepe
leventk@sherinstitute.com

[1] Sher Institute for Reproductive Medicine, Las Vegas, NV, USA

[2] Department of Medical Genetics, University of Bursa, Bursa, Turkey

Introduction

Identification of aneuploid human embryos produced in vitro by recent advanced molecular genetic testing remains challenging because of high rates of mosaicism, atypical cell division, cellular fragmentation, sub-chromosomal instability, and micro-/multi-nucleation. Additionally, several of these processes occur in vivo following natural human conception, suggesting that they are not simply a consequence of culture conditions.

Numerical chromosome errors are known to be common in early human embryos and probably contribute significantly to early pregnancy loss and implantation failure in IVF patients [1,2]. It is estimated that >50 % of all embryos contain at least one aneuploid cell [3]. Many of these aneuploidies are rarely, if ever, seen in later stage fetuses [4]. Molecular techniques have been utilized during IVF cycles to determine embryo ploidy including fluorescence in situ hybridization (FISH) [11], comparative genomic hybridization (CGH) [5,6], digital polymerase chain reaction (dPCR) [7], single-nucleotide polymorphism (SNP) array [8], real-time quantitative PCR (qPCR) [9], and next-generation sequencing (NGS) [10]. These

technologies are reputed to provide enhanced sensitivity to detect the most competent embryo(s) for transfer.

Recently, questions regarding the reliability of preimplantation genetic diagnosis (PGD)/preimplantation genetic screening (PGS) testing for in vitro grown human embryos have been raised because of the chromosomal mosaicism [11,12]. Mosaic embryos, which are comprised of both euploid and aneuploid cells, are difficult to identify as such, and when they are identified, they are traditionally not transferred because of their ambiguous viability [13]. Nonetheless, such embryos may remain the sole recourse for transfer in patients with very poor fertility prognoses [14]. The published literature suggests that older women do not benefit from PGS, and this makes sense in that most embryos in these patients will be aneuploid and additional screening seems somewhat redundant [15,16]. Moreover, if these patients produce mosaics, they are often discarded, and this may be counterproductive as it has since been revealed that some mosaic embryos can produce healthy babies [16].

These and some of our controversial data prompted us to assess PGD/PGS embryo testing results when the abnormal results were presented. Therefore, we examined the data accrued from our centers in which sequential biopsies, taken from the same embryo, were sent to two different established genetics laboratories.

Material and methods

All biopsies were trophectoderm biopsies that were obtained from day 5 or 6 expanded blastocysts by the same embryologists. After biopsy, blastocysts were cryobanked via vitrification [17]. The biopsy samples were analyzed using aCGH (Reprogenetics, Livingston, NJ) or SNP array (Natera, San Carlos, CA) in the first round. Embryos deemed to have an abnormal chromosomal content by virtue of the first PGS laboratory were subsequently thawed, re-biopsied after demonstrating viability, and then re-vitrified. These second biopsies were sent to a different genetic diagnostic laboratory and analyzed by either SNP array or NGS (Progenesis, La Jolla, CA).

For aCGH analysis, TE cell samples were amplified using the SurePlex DNA Amplification System (Illumina, Inc., San Diego, CA, USA), according to the manufacturer's protocol. Whole genome amplification (WGA) products were processed with 24sure V3 microarrays (Illumina, Inc.), according to the manufacturer's protocol.

For SNP genotyping, amplified single cells were genotyped using Illumina Infinium II genome-wide genotyping microarrays.

For NGS, WGA was performed using PicoPLEX® Technology according to the manufacturer's instructions (Rubicon Genomics, Ann Arbor, MI, USA). The data analysis was done by 100,000 to 200,000 reads and approximately 200-bp size per amplicon, totaling 20 million to 40 million base pairs per sample. All reads were filtered for polyclonals and aligned to the human genome database using Torrent Suite™ Software for Sequencing Data Analysis. Quality reads were scored for aneuploidy using Ion Reporter™ software (Thermofisher Scientific, Waltham, MA, USA).

Results

A total of 37 cryopreserved aneuploid blastocysts were maintained at the SIRM centers until the second biopsy was carried out by standard laser-assisted technique. Specimens were coded according to the instructions provided by the testing laboratories and then sent it in for analysis.

Outcome comparison between PGS testing methods

The tabulated results were summarized in Table 1. The distribution of 37 abnormal embryos was as follows: 4 embryos were initially analyzed by aCGH then with SNP array; 6 of them were initially with SNP array then NGS; 27 of them were initially analyzed by aCGH then with NGS. Only 3/27 (11 %) of the embryos demonstrated complete compatible results between both aCGH and NGS. Importantly, 9/27 (33 %) of the originally reported aneuploid embryos, upon repeat assessment, were found to be euploid. On the other hand, the concurrence for aCGH and SNP array were 0 % (0/3). Originally reported abnormal embryos with aCGH, on repeat assessment with SNP array were found to be 100 % (3/3) normal. The agreement between SNP array and NGS were 50 % (3/6), and 17 % (1/6) of these abnormal embryos tested normal with NGS. When 27 of CGH abnormal embryos were retested, 11 of them (41 %) were determined completely normal by NGS. The concordance between aCGH and NGS was 4 % (1/27). Additionally, three embryos which demonstrated abnormal gender karyotypes (XXY) with aCGH were found to be completely normal by NGS. Furthermore, in three instances, the opposite gender was obtained with NGS when aCGH abnormal embryos were reanalyzed.

The data also demonstrated a high rate of mosaicism (44 % (16/36)) when two consecutive biopsy samples obtained from a same blastocyst.

Discussion

The main goal of PGS has always been the improvement of IVF success rates. In the rapidly evolving field of reproductive medicine, the introduction of new technologies or treatments

Table 1 Comparison of embryo ploidy between testing methods

Embryo ID	Biopsy #	Sample #1	Gender	Original Testing	Sample #2	Gender	Repeat testing
SH-1-2	2	Tri 18	XY	aCGH	NL	XY	NGS
SH-1-8	2	Tri 21	XX	aCGH	NL	XX	NGS
SH-1-15	2	Mono 18	XX	aCGH	NL	XX	NGS
SH-2-1	2	Tri 16	XXY	aCGH	Tri 16	XY	NGS
SH-2-8	2	Tri 15, 17	XY	aCGH	Tri 15	XY	NGS
SH-3-1	2	Mono 21, Tri 13	XX	aCGH	NL	XX	NGS
SH-3-2	2	Tri 15, 18	XY	aCGH	NL	XY	NGS
SH-4-18	2	Mono 4	XX	SNP a	Mono 4	XX	NGS
SH-4-19	2	Tri 1	XY	SNP a	Tri 1	XY	NGS
SH-5-1	2	Mono 12	XX	aCGH	NL	XX	SNP a
SH-5-2	2	Tri 9, 13, 14, 17	XX	aCGH	NL	XX	SNP a
SH-5-4	2	No Result	N/A	aCGH	No Result	N/A	SNP a
SH-5-6	2	Tri 2, 20	XY	aCGH	NL	XY	SNP a
SH-6-2	2	Mono 21	XY	aCGH	Mono 21	XY	NGS
SH-6-4	2	Tri 9, 13	XY	aCGH	Tri 13	XY	NGS
SH-6-7	2	Mono 3, Tri 20, 21	XY	aCGH	NL	XY	NGS
SH-6-12	2	Partial Mono 18	XX	aCGH	Complex ABN	XX	NGS
SH-6-13	2	Tri XXY	XXY	aCGH	NL	XY	NGS
SH-6-15	2	Mono 7, 20	XY	aCGH	Tri 7, 20	XY	NGS
SH-6-17	2	Mono 8, Tri 12, 16, 22	XY	aCGH	Tri 8, 16	XY	NGS
SH-6-11	2	Mono 3, Tri 20, 21	XY	aCGH	Mono 3	XY	NGS
SH-7-1	2	Mono 13	XY	SNP a	Tri 14	XX	NGS
SH-7-2	2	Tri/polysomy 14	XX	SNP a	Mono 13	XY	NGS
SH-7-3	2	Tri 11,12,15,17,21	XXY	aCGH	Tri 7,8,10,11	XX	NGS
SH-7-4	2	Tri 5,6,11,17,18,21	XX	aCGH	Tri 5,13,17	XX	NGS
SH-7-5	2	Tri 21	XX	aCGH	Tri 2	XX	NGS
SH-7-6	2	Tri 2	XX	aCGH	Complex ABN	XX	NGS
SH-7-7	2	Del/Dup 5	XX	SNP a	NL	XY	NGS
SH-7-8	2	Tri/polysomy 13	XX	SNP a	Tri 13	XX	NGS
SH-8-1	2	Complex ABN	XY	aCGH	Complex ABN	XY	NGS
SH-8-2	2	Complex ABN	XY	aCGH	XO	XO	NGS
SH-8-3	2	Mono 13	XX	aCGH	Mono 13	XX	NGS
SH-8-4	2	Partial Tri 5	XY	aCGH	NL	XY	NGS
SH-8-5	2	Mono 16, Tri 3	XY	aCGH	NL	XY	NGS
SH-8-6	2	Tri 21	XX	aCGH	NL	XX	NGS
SH-8-7	2	Tri 13, 20	XX	aCGH	NL	XX	NGS
SH-8-8	2	Tri 13, 21	XX	aCGH	NL	XX	NGS

is common practice. In an ideal world, such an introduction is preceded by scientific research which confirms the efficacy and safety of the intervention. Before its wide adoption clinically, any innovative technique should be validated by sufficient research as reliable, safe, and technically feasible.

Human race is considered to be a poor reproducer because of having very high natural abortion rate and having a number of chromosomal abnormalities during natural conception cycles. A study conducted on normal fertile couples with a risk for inherited genetic diseases that only 9 % of all generated

IVF embryos had a normal chromosomal complement in all blastomeres and that nearly half of the embryos had no normal blastomeres [18]. Aneuploidy occurs because of inappropriate attachment of chromosomes to the mitotic spindle, partial inactivation of spindle checkpoint proteins or the amplification of centrosomes [19]. New array-based methods more recently next-generation sequencing [20] allowed screening of genome-wide copy number and specifically mosaicism and uniparental disomies in most cleavage-stage embryos but also frequent segmental deletions, duplications, and amplifications. However, several challenges remain, including the potential detriment of the embryo following biopsy for PGS [21], chromosomal mosaicism between various cells or cell populations [22], and the effects of sub-chromosomal aberrations [23] on subsequent development. Recent identification of common genetic variants, or DNA sequence variation, near genes in patients that are at high-risk for producing aneuploid embryos further complicates the already complex process of pre-implantation chromosomal instability [24].

Over the past decade, a large body of literature has been amassed related to genotype errors for microarrays. In particular, nondifferential genotyping errors, that is, errors that are the result of a random process unrelated to the phenotype, decrease power of the test.

With the advent of next-generation sequencing (NGS), multimarker analysis methods have increased in popularity. Recent papers demonstrate similar error rates are true for multimarker tests as well. To date, large error rates have been observed for sequence data [14–16], much larger than were typical in microarrays [17]. Thus, there is the potential for substantial power loss and inflated errors for multimarker tests involving NGS data.

In our data set, the chromosomal evaluation by two different genetic testing methods (aCGH vs NGS) in the same embryo were corresponding in only 1/27 (4 %) embryos. Indeed, 11/27 embryos (41 %) in initial evaluation reported to be aneuploid, on repeat evaluation by NGS were reported to be normal embryos. An additional 8/27 (30 %) embryos were mosaic when aCGH was abnormal but NGS was normal. Besides, three embryos which demonstrated abnormal gender karyotypes (XXY) with aCGH were found to be completely normal by NGS. Likewise, in three instances, the opposite gender was obtained with NGS when aCGH abnormal embryos were reanalyzed.

Blastomere biopsy is presently the most adapted type of biopsy for PGS/PGD. TE biopsies from day 5 and 6 blastocysts enable detection of both mitotic and meiotic aneuploidies and allow cleavage-stage embryos the opportunity to "self-correct" therefore improving the efficiency. However, comparisons of aneuploidy rates with blastomere biopsies versus TE biopsies are conflicting. Whereas studies using fluorescence in situ hybridization (FISH) have found similar rates of aneuploidy in cleavage-stage and TE measurements [25], studies using comparative genome hybridization (CGH) found low rates of aneuploidy in blastocysts compared with cleavage-stage measurements [26].

In a recent study, Johnson et al. [27] and Capalbo et al. [28] demonstrated mosaicism were low in human blastocysts and the discordance rate between ICM and TE cells were negligible. These studies contradict with our data presented here, which showed high discordance rate in a consecutive biopsies from a same embryo. The abovementioned studies represents a significant underestimation is suggested by an earlier study of Fragouli et al. [29] who reported that among 52 investigated blastocyst-stage embryos, 32.4 % were mosaic, 30 % uniformly aneuploid, and 42.3 % uniformly euploid. The difference could be because of employing the same molecular technique between TE and ICM cells in case of Johnson et al., and employing only nine chromosome FISH analysis in Capalbo et al., whereas we tried to establish concordance between two different molecular platform. Although the true mosaicism rate in human embryos is not known, it was estimated around in the 30–50 % range, depending on female age and functional ovarian age [12]. Orvieto et al. [30] biopsied the top quality blastocysts three different spots and tested them by NGS. The results revealed 20.8 % inconclusive results and 16.6 % embryonic mosaicism. Their overall results had 35.7 % mosaicism or inconclusive results. Our observations demonstrated slightly higher rate of mosaicism (44 %) in the TE cells when two consecutive biopsied were carried over on the same blastocysts. We also observed nondisjunctional chromosomal error between those two samples. The source of nondisjunctional errors is challenging to be determined in this data setting since it could be from meiosis I, II, or during the mitosis.

The data presented here adds more complexity into the existing PGS debate and offers further evidence that the increasing unselective utilization of PGS could be harmful for a certain group of patients. There are very few studies directly comparing the specific laboratory techniques for assessing ploidy. Future studies are warranted to determine if any platforms are superior to each other and the extent of embryonic mosaicism in different patient populations. Furthermore, the number of genetic testing platforms and companies has grown notably, operating with little or no regulatory oversight. Interlaboratory platform differences may be significant contributors to the overall concerns related to the PGS testing. We believe that it is imperative for those involved such as genetic testing companies, clinicians, and professional organizations to seek and establish a system by which the performance/accuracy of these tests/companies are independently verified on a regular basis. Such a system would builds the confidence in the technology/industry and benefits all parties involved. As it is suggested by the ASRM practice committee, "other important considerations about PGS that must be addressed by

further research include cost effectiveness, the role and effect of cryopreservation, time to pregnancy, cumulative success rates over time, and total reproductive potential per intervention." We must also acknowledge that it is currently not established whether the lack of concordance was a result of a second biopsy with a different cohort of cells (i.e., mosaicism) or inherent lack of concordance between platforms or human error. We urge all involved parties to review the outcomes obtained via PGS and carefully reassess its applicability and validity.

References

1. Mastenbroek S, Twisk M, van Echten-Arends J, Sikkema-Raddatz B, Korevaar JC, Verhoeve HR, et al. In vitro fertilization with preimplantation genetic screening. N Engl J Med. 2007;357:9–17.
2. Gleicher N, Kushnir VA, Barad DH. Preimplantation genetic screening (PGS) still in search of a clinical application: a systematic review. Reprod Biol Endocrinol. 2014;12:22.
3. Barzrgar M, Gourabi H, Valojerdi MR, Yazdi PE, Baharvand H. Self-correction of chromosomal abnormalities in human preimplantation embryos and embryonic stem cells. Stem Cells Dev. 2013;22:2449–56.
4. Kalousek DK, Dill FJ. Chromosomal mosaicism confined to the placenta in human conceptions. Science. 1983;221:665–7.
5. Wells D, Sherlock JK, Handyside AH, Delhanty JD. Detailed chromosomal and molecular genetic analysis of single cells by whole genome amplification and comparative genomic hybridisation. Nucleic Acids Res. 1999;27(4):1214–8.
6. Munné S, Wells D, Cohen J. Technology requirements for preimplantation genetic diagnosis to improve assisted reproduction outcomes. Fertil Steril. 2010;94:408–30.
7. Evans MI, Wright DA, Pergament E, Cuckle HS, Nicolaides KH. Digital PCR for noninvasive detection of aneuploidy: power analysis equations for feasibility. Fetal Diagn Ther. 2012;31(4):244–7.
8. Treff NR, Levy B, Su J, Northrop LE, Tao X, Scott Jr RT. SNP microarray-based 24 chromosome aneuploidy screening is significantly more consistent than FISH. Mol Hum Reprod. 2010;16(8):583–9.
9. Werner MD, Scott Jr RT, Treff NR. 24-chromosome PCR for aneuploidy screening. Curr Opin Obstet Gynecol. 2015;27(3):201–5.
10. Yan L, Huang L, Xu L, Huang J, Ma F, Zhu X, et al. Live births after simultaneous avoidance of monogenic diseases and chromosome abnormality by next-generation sequencing with linkage analyses. Proc Natl Acad Sci U S A. 2015;112(52):15964–9.
11. Esfandiari N, Bentov Y, Casper RF. Trophectoderm biopsy for aneuploidy screening using different platforms and conflicting test results. Abstract O-034, Annual Meeting of ESHRE, Munich June 27- July 2, 2014.
12. Tiegs AW, Hodes-Wertz B, McCulloh DH, Grifo J. Clinical error rate of array comparative genomic hybridization (ACGH) in euploid blastocysts. Fertil Steril. 2015;104 Suppl 3:e275.
13. Greco E, Minasi G, Fiorentino F. Healthy babies after intrauterine transfer of mosaic aneuploid blastocysts. N Engl J Med. 2015;373:2089–90.
14. Gleicher N, Vidali A, Braverman J, Kushnir VA, Albertini DF, Barad DH. Further evidence against use of PGS in poor prognosis patients: report of normal births after transfer of embryos reported as aneuploid. Fertil Steril. 2015;104 Suppl 3:e9.
15. Conlin LK, Thiel BD, Bonnemann CG, Medne L, Ernst LM, Zackai EH, et al. Mechanisms of mosaicism, chimerism and uniparental disomy identified by single nucleotide polymorphism array analysis. Hum Mol Genet. 2010;19:1263–75.
16. Mastenbroek S, Repping S. Preimplantation genetic screening: back to the future. Hum Reprod. 2014;29:1846–50.
17. Keskintepe L, Sher G, Machnicka A, Tortoriello D, Bayrak A, Fisch J, et al. Vitrification of human embryos subjected to blastomere biopsy for pre-implantation genetic screening produces higher survival and pregnancy rates than slow freezing. J Assist Reprod Genet. 2009;26(11–12):629–35.
18. Vanneste E, Voet T, Le Caignec C, Ampe M, Konings P, Melotte C, et al. Chromosome instability is common in human cleavage-stage embryos. Nat Med. 2009;15(5):577–83.
19. King RW. When 2+2=5: the origins and fates of aneuploid and tetraploid cells. Biochim Biophys Acta. 2008;1786(1):4–14.
20. Fiorentino F, Bono S, Biricik A, Nuccitelli A, Cotroneo E, Cottone G, et al. Application of next-generation sequencing technology for comprehensive aneuploidy screening of blastocysts in clinical preimplantation genetic screening cycles. Hum Reprod. 2014;29(12):2802–13.
21. Treff NR, Fedick A, Tao X, Devkota B, Taylor D, Scott Jr RT. Evaluation of targeted next-generation sequencing-based preimplantation genetic diagnosis of monogenic disease. Fertil Steril. 2013;99(5):1377–84.
22. Baart EB, Martini E, van den Berg I, Macklon NS, Galjaard RJ, Fauser BC, et al. Preimplantation genetic screening reveals a high incidence of aneuploidy and mosaicism in embryos from young women undergoing IVF. Hum Reprod. 2006;21(1):223–33.
23. Vanneste E, Voet T, Melotte C, Debrock S, Sermon K, Staessen C, et al. What next for preimplantation genetic screening? High mitotic chromosome instability rate provides the biological basis for the low success rate. Hum Reprod. 2009;24(11):2679–82.
24. McCoy RC, Demko ZP, Ryan A, Banjevic M, Hill M, Sigurjonsson S, et al. Evidence of selection against complex mitotic-origin aneuploidy during preimplantation development. PLoS Genet. 2015;22:11(10).
25. Sandalinas M, Sadowy S, Alikani M, Calderon G, Cohen J, Munné S. Developmental ability of chromosomally abnormal human embryos to develop to the blastocyst stage. Hum Reprod. 2001;16(9):1954–8.
26. Schoolcraft WB, Katz-Jaffe MG, Stevens J, Rawlins M, Munne S. Preimplantation aneuploidy testing for infertile patients of advanced maternal age: a randomized prospective trial. Fertil Steril. 2009;92(1):157–62.
27. Johnson DS, Gemelos G, Baner J, Ryan A, Cinnioglu C, Banjevic M, et al. Preclinical validation of a microarray method for full molecular karyotyping of blastomeres in a 24-h protocol. Hum Reprod. 2010;25(4):1066–75.
28. Capalbo A, Wright G, Elliott T, Ubaldi FM, Rienzi L, Nagy ZP. FISH reanalysis of inner cell mass and trophectoderm samples of previously array-CGH screened blastocysts shows high accuracy of diagnosis and no major diagnostic impact of mosaicism at the blastocyst stage. Hum Reprod. 2013;28(8):2298–307.
29. Fragouli E, Alfarawati S, Spath K, Tarozzi N, Borini A, Wells D. The developmental potential of mosaic embryos. Fertil Steril. 2015;104 Suppl 3, e96.
30. Orvieto R, Shuly Y, Brengauz M, Feldman B. Should preimplantation genetic screening be implemented to routine clinical practice? Gynecol Endocrinol. 2016;12:1–3.