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Preimplantation Microarray Analysis (PMA) is a Robust Technique that Allows for Aneuploidy Screening of all 24 Chromosomes with a Lower Misdiagnosis Rate than FISH Based Methodologies

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INTRODUCTION

The preparation of metaphase spreads is an essential part of chromosome analysis. This requires tissue culture to obtain multiple cells that are actively dividing that can be arrested in the metaphase stage of the cell cycle. Fluorescence in situ hybridization (FISH) with chromosome specific probes can enumerate individual chromosomes in an interphase cell. However, the total number of chromosomes that can be enumerated at once is limited. SNP oligonucleotide microarray analysis (SOMA) is a DNA based cytogenetic techniques that allows for the identification of chromosomal gains and losses without the need for cell culturing. Imbalances in chromosomal material are detected in a single-step scan of the entire genome. Since it is not feasible to culture and perform cytogenetic analysis on single blastomeres that have been obtained following embryo biopsy, SOMA presents itself as an attractive technique for identifying aneuploidy in single human cells. The blend of whole-genome amplification & microarray technology may provide an attractive solution to the limitations of current preimplantation FISH-based aneuploidy screening strategies.

MATERIALS & METHODS

Study Embryos: All studies were performed using IRB approved protocols and appropriate patient consent.

Study 1: Eight arrested cleavage stage embryos were biopsied into individual cells, randomized into even groups & blindly assigned for further analysis by FISH (n=51) or SOMA (n=52). In order to demonstrate the accuracy of SOMA, we compared the consistency of the results in embryos analyzed both by a 9 chromosome FISH panel & by SOMA. For each method, results were evaluated for mosaicism, number of unique chromosomal compliments, and number of individual chromosomes with abnormal copy numbers via paired analyses.

Study 2: Whole genome amplification (WGA) was performed on 10 individual blastomeres derived from 10 different day 3 arrested embryos. DNA aliquots from each blastomere were blinded and then subjected to CGH and PMA by SOMA. The results from each method were tabulated and compared following decoding.

Study 3: 4304 embryos that underwent PGD by FISH (fPGD) were compared to 505 embryos evaluated by microarray PGD (PMA). Phase I: The prevalence of abnormality in fPGD and PMA results were calculated for each chromosome. The overall diagnosis which would Then be assigned to each embryo were compared using contingency tables. Phase II: Using the PMA data as a standard, misdiagnoses rates for 5, 7, and 9 probe fPGD were calculated. Potential over-Diagnoses with fPGD were calculated by contrasting the overall abnormality rate per embryo in fPGD vs PMA - not by simply adding individual errors. The under-diagnoses errors were estimated by looking at the specific subset of PMA evaluated embryos which were normal for the chromosomes which would have been evaluated by fPGD, but were abnormal for one of the other chromosomes. The overall misdiagnoses rate was obtained by adding the two potential sources of error.

WGA: WGA was performed using a modification of the GenomePlex WGA4 system (Sigma-Aldrich).

CGH Analysis: WGA-DNA was labeled using nick-translation. CGH probes were prepared and washed as described by Kallioniemi et al (1994) and Levy et. al (1998). The florescence ratios (green/red) for at least 10 of each autosome and 7 of each sex chromosome were obtained per slide. The genetic diagnosis was assigned using results obtained at the 99.9-99.99% confidence interval (CI) and the sex was assigned using the X and Y chromosome results at the 99.5% CI.

Preimplantation Microarray Analysis (PMA): PMA by SOMA was performed on a genome-wide 250K NspI SNP genotyping microarray (Affymetrix). Copy number imbalances (genetic diagnosis) were determined using the Chromosome Copy Number Analysis Tool (CNAT) version 4.0 (www.affymetrix.com). Validation and clinical predictive value of this method have been previously presented (ASRM 2007 #O-1, ASRM 2008 #O-62). The sex was assigned by a combination of the microarray X chromosome profiles and a separate Y-specific PCR assay.

RESULTS

Study 1: 2 cells from the PMA group did not amplify. 8 cells from the fPGD had uninterpretable results. Mosaicism was less common with PMA (2/8) than fPGD (8/8) (P<0.05). When mosaicism was found, the number of distinct diagnoses was greater with fPGD (2-6) than PMA (2-3) (P<0.01). In 2 embryos where mosaicism was found with PMA, the mosaicism made sense. In embryo 7, 3 cells were 46,XY, 1 was 45,XY-13, and 2 were 47,XY+13. In embryo 8, 4 cells were 45,X and 1 was 47,XXX. In each case, a single chromosome was involved

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fPGD results showed a 100% mosaicism rate. Only 1 embryo's fPGD results potentially made sense; 3 cells were 45,X and 1 was 44,X-13 (monosomy X - meiotic error; monosomy 13 - mitotic error). The fPGD results in the other 7 embryos varied significantly and were not biologically logical. More chromosomes were called aneuploid with fPGD (33-55%; 3 to 5 of 9) than with PMA (0-4%; 0 to 1 of 24) (P<0.05).

8 Embryos Evaluated - 103 total cells tested	
fPGD – 51 cells	PMA – 52 cells
Prevalence of No Interpretable Result	
fPGD – 8 of 51 (16%)	PMA – 2 of 52 cells (4%) *
Prevalence of Mosaicism	
fPGD – 100%	PMA – 25%

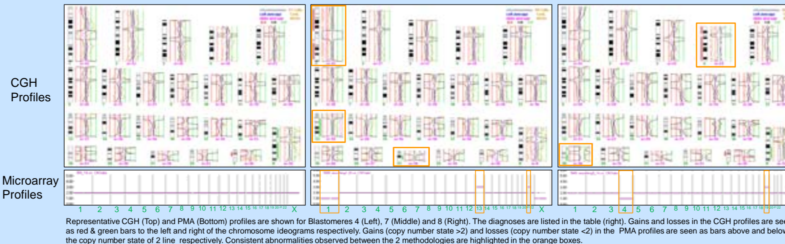
* PMA with significantly fewer uninterpretable results P<0.05

Table 1 – Comparison of Assigned Genetic Diagnoses. This table lists the number of different unique genetic diagnoses assigned by either fPGD or by PMA. Note that fPGD only had 9 chromosomes but still managed to demonstrate a 100% mosaicism rate with an average of 3.4 different assigned diagnoses per embryo. In contrast, PMA evaluates 24 chromosomes and still only found a 25% mosaicism rate. In the two cases where mosaicism occurred, the different genetic diagnoses involved changes in a single chromosome. For example, in embryo 8, some cells had monosomy X while the others had trisomy X. The abnormalities were complimentary. No such relationships were identified in the cells analyzed by fPGD.

TABLE 1

	Type of PGD			
	fPGD		PMA	
Embryo	Number of Unique Genetic Diagnoses	Actual Genetic Results	Number of Unique Genetic Compliments	Actual Genetic Results
1	4 (6 cells)	Null: 13; Mono: 15, 21, 22 Mono 18 Mono 17 Normal Female	1 (7 cells)	46,XX
2	5 (7 cells)	Null: 15, 18, 21 Mono: 17 Mono 17 Mono 18 Mono 21 Normal Male	1 (7 cells)	46,XY
3	3 (5 cells)	Mono: 16, 18; Tri: 13 Mono 13 Normal Male	1 (6 cells)	46,XY
4	3 (5 cells)	Mono 16 Mono 18 Tri: 13	1 (8 cells)	46,XY
5	4 (6 cells)	Mono: 15; Tri: 13, 21 Tri: X Tri: 18 Normal Female	1 (7 cells)	47,XX+18
6	4 (7 cells)	Mono: 21 Tri: 13 Mono: 17 Tri: 18 Normal Male	1 (6 cells)	45,X
7	3 (4 cells)	Mono 13, Mono: 15; Tri 18 Normal Male	3 (6 cells)	45,XY-13 46,XY 47,XY+13
8	2 (4 cells)	Tri 16 Normal Female	2 (5 cells)	45,X 47,XXX

Study 2: The sex assignment matched for all specimens and 90% concordance was observed when comparing the overall diagnosis (Table 2 below). The overall concordance between methods with respect to the status of each chromosome (n=240 chromosomes) was 97.5%. The discrepancy in blastomere 6 involved a small imbalance of the short arm of chromosome 12. The PMA profile did in fact show evidence of a gain of 12p but thresholds for partial aneuploidy in PMA have not yet been established. In blastomere 10, the diagnosis of trisomy 16 was consistent between the 2 methods but trisomy 21 was discrepant.



Representative CGH (Top) and PMA (Bottom) profiles are shown for Blastomeres 4 (Left), 7 (Middle) and 8 (Right). The diagnoses are listed in the table (right). Gains and losses in the CGH profiles are seen as red & green bars to the left and right of the chromosome ideograms respectively. Gains (copy number state >2) and losses (copy number state <2) in the PMA profiles are seen as bars above and below the copy number state of 2 line respectively. Consistent abnormalities observed between the 2 methodologies are highlighted in the orange boxes.

Blastomere ID	Sex Assignment		Overall Diagnosis		Specific Genetic Diagnosis	
	CGH	PMA	CGH	PMA	CGH	PMA
1	Female	Female	Abnormal	Abnormal	Partial Monosomy 19q Trisomy 22	Monosomy 19 Trisomy 22
2	Female	Female	Normal	Normal	Normal	Normal
3	Female	Female	Normal	Normal	Normal	Normal
4	Female	Female	Normal	Normal	Normal	Normal
5	Male	Male	Normal	Normal	Normal	Normal
6	Male	Male	Abnormal	Normal	Partial Trisomy 12p	Normal
7	Male	Male	Abnormal	Abnormal	Monosomy 1 Trisomy 13 Trisomy 21	Monosomy 1 Trisomy 13 Trisomy 21
8	Female	Female	Abnormal	Abnormal	Monosomy 4 Trisomy 19	Monosomy 4 Trisomy 19
9	Male	Male	Abnormal	Abnormal	Complex Aneuploid *	Complex Aneuploid *
10	Male	Male	Abnormal	Abnormal	Trisomy 16 Trisomy 21	Trisomy 16

Study 3: There were no differences in the patients ages between the groups. Phase I: Abnormality rates with fPGD were markedly higher than those with PMA (P<1x10-6). The rate of any abnormality within those 9 chromosomes (thus assigning an abnormal diagnosis to the embryo) was also markedly higher for fPGD (P<1x10-6). The assignment of abnormal diagnosis with fPGD was higher than with PMA even after abnormalities of all 24 chromosomes were considered (P<1x10-6). Phase II: The estimated over-diagnosis rate for fPGD varied from 37% to 40%. The under-diagnosis rate was 12% to 25%. The latter estimate is likely low, since a disproportional increase in over-diagnoses would be expected as probes were added to fPGD.

Abnormality Rates with fPGD and PMA

Chromosome	f-PGD	PMA
13	27.8	1.0
15	25.6	5.0
16	29.6	9.3
17	19.9	3.0
18	28.6	5.1
21	29.0	5.1
22	31.9	5.9
Sex Chromosomes	22.6	7.7
Whole Embryo	69.5	29.3

Estimation of fPGD Error Rate

Number of FISH Probes	fPGD Abnormal (%)	PMA Abnormal (%)	FISH Overdiagnosis (Dx abnormal when actually normall)	% FISH Underdiagnosis (failed to identify abnormals in chromosomes not tested)	Overall FISH Misdiagnosis Rate (%)
5	53.7	16.4	37.3	25.6	62.9
7	65.1	25.9	39.2	16	55.3
9	69.5	29.3	40.2	12	52.3

DISCUSSION & CONCLUSION

Clinical validation of new technologies is particularly challenging when dealing with single cell diagnostics. The introduction of FISH based PGD into the clinical arena offered great potential for enhancing embryo selection. Unfortunately, clinical results have not demonstrated the theoretical improvements which should accompany elimination of chromosomally abnormal embryos from the transfer "pool". One option for looking at the precision of fPGD on blastomeres from day 3 embryos would be to dissociate an embryo which has been discarded for research and to randomly assign each cell to analysis by either FISH or a another method such as microarray analysis (PMA using SOMA). The overall prevalence of mosaicism between the cells analyzed by each technique should be similar. Additionally, the specific chromosomes involved in any abnormality should be complimentary. Any significant disparity in the consistency of genetic results with one technique relative to the other would strongly suggest that the one is not providing a reliable result. Further analysis of the results to see if they are internally consistent (i.e. the same chromosome involved in the mosaicism – trisomy in one cell and monosomy in another) could be used to identify the technique which is likely more reliable. The inconsistencies observed in the fPGD results in study 1 point to likely intrinsic inaccuracies associated with single cell FISH diagnostics. In contrast, the high level of consistency in study 2 of the results obtained by both CGH and microarray methods indicates that these assays are robust and likely to offer a superior means for aneuploidy screening in single cells compared to current FISH-based techniques. Since high resolution CGH is a technically challenging and tedious procedure, PMA may be better suited for aneuploidy screening of all 24 human chromosomes in a clinical setting. In a study analyzing a large cohort of embryos by two different techniques, one would expect similar abnormality rates if there are no differences in the ages of the patients being analyzed by the two different methodologies. The results of study 3 coupled with the consistent diagnostic results of PMA as demonstrated in studies 1 & 2, indicate that fPGD provides significant misdiagnoses with over-diagnoses of evaluated chromosomes and omission of abnormalities in those not evaluated. This may partially explain the failure of fPGD to produce meaningful improvements in clinical outcomes for IVF patients.