Evaluation of KaryoLite™ BACs on Beads™ assay for preimplantation genetic testing for aneuploidy in blastocyst

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ABSTRACT

Preimplantation genetic testing for aneuploidy (PGT-A) is used to screen chromosomal abnormalities in embryos. Currently, a new molecular karyotyping platform, KaryoLite™ bacterial artificial chromosomes (BAC)s on Beads™ (KL-BoBs) assay was developed to detect aneusomy for any chromosomes. Here, we present an evaluation of KL-BoBs for a routine diagnostic test in a clinical setting for PGT-A by comparison with array-based comparative genomic hybridization (aCGH) result. A total of 39 samples from 14 frozen blastocysts were obtained from 3 patients. The frozen blastocysts were studied in the first biopsy, and aCGH was tested parallelly to detect chromosomal abnormalities in biopsied trophectoderm (TE). Consistency of the results obtained from biopsied inner cell mass (ICM) and TE was 98.2%. The biopsied TE in human blastocysts can predict the chromosome information in ICM. We found that 36.36% of TE from the same abnormal blastocysts had mosaicism. According to the limitation of KL-BoBs, polyploidy and structural abnormalities were unable to be detected. The results from KL-BoBs had concordance with those from aCGH technique to detect chromosomal abnormalities for PGT-A. Consistency of biopsied ICM and TE was 98.2%, which indicated that biopsied TE in human blastocysts could predict chromosome information in ICM.

Keywords: KaryoLite™ BACs on Beads™; PGT-A; blastocyst; trophectoderm; embryo

INTRODUCTION

Preimplantation genetic testing for aneuploidy (PGT-A) is used for patients with recurrent pregnancy loss, advanced maternal age, and repeated *in vitro* fertilization (IVF) cycle failure to screen embryos to evaluate chromosomal abnormalities. Performing PGT-A and transferring euploid embryos leads to increased pregnancy rates and live birth rates (Chang *et al.*, 2011; Huang *et al.*, 2011; Homer, 2019). However, PGT-A requires accessing DNA from embryos before six days of conception when implantation occurs. Besides, the most commonly applied invasive test is PGT-A of the chromosomal constitution of polar bodies, blastomeres (3rd-day cleavage embryos), and trophectoderm cells (5th to 6th-day blastocyst) (Harton *et al.* 2010)

Recently, array comparative genomic hybridization (aCGH) and next-generation sequencing (NGS) which requires whole genome amplification (WGA) step to amplify a single-cell DNA content before genetic analysis was included in PGT-A clinical practice (Cinnioglu *et al.*, 2019). The advantage of aCGH and NGS is the ability to detect all chromosomes with high resolution, but the cost is more expensive than other techniques (Harper *et al.* 2010; 2012).

KaryoLite™ bacterial artificial chromosome (BAC)s on Beads™ (KL-BoBs) is a molecular karyotyping technology which contains low-resolution coverage of all chromosomes and provides dosage information about proximal and terminal regions of each chromosome arm. Additionally, there are 3 to 4 beads per chromosome, and each bead contains three neighboring BAC probes to broaden the hybridization target region. Aneusomy detection with the KL-BoBs assay is performed by comparing the tested sample to reference DNA samples (Shaffer *et al.* 2011; Grati *et al.* 2013; Paxton *et al.* 2013).

In this study, we report an evaluation of KL-BoBs for PGT-A by comparing it with aCGH result. Moreover, we studied the consistency result of trophectoderm (TE) with abnormal blastocysts and analyzed the consistency results of abnormal blastocysts in the inner cell mass (ICM) and TE.

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MATERIALS AND METHODS

DNA samples

For the validation study, 45 samples were obtained from 26 patients from the Faculty of Medicine Ramathibodi Hospital, Mahidol University. The samples were amniotic fluids (AF) (n = 37), oocytes (n = 2), blastomeres (n = 5) and blastocyst (n=1). Cytogenetic findings were known in all amniotic fluid samples, but not the others. Amniotic fluid cell samples were divided into 5 groups: trisomy 18 (n = 6), trisomy 21 (n = 11), triple X (n = 6), 46, XX (n =7) and 46, XY (n = 7).

For the clinical samples, 39 samples from 14 frozen blastocysts were obtained from 3 patients. The frozen blastocysts were studied in the first biopsy, and aCGH was tested in parallel to detect chromosomal abnormalities in the biopsied TE. The second biopsy was performed in TE (n = 28) and ICM (n = 11) to detect aneuploidy and study chromosome mosaicism status in TE by comparing the results between KL-BoBs and aCGH. Specimen collection for this study was approved by Ramathibodi Hospital Ethics Committee (No. ID 10–57–51).

Sample preparation

Amniotic fluid cells were washed with 1X PBS to minimize carryover of external DNA contaminants, transferred into 0.2 ml microcentrifuge tube and stored at -80°C until use. Embryo biopsy was performed in TE and ICM of the frozen blastocyst by laser techniques (Hamilton Thorne, USA). Then they were washed with 1X PBS, transferred into a 0.2 ml microcentrifuge tube with 1 µl 1X PBS and store at -80°C until use.

Quantity and quality assessment

Genomic DNA was amplified using a picoplex WGA kit (Rubicon Genomics, USA) to produce 2 to 5 micrograms of amplified DNA within 3 hours. The amplified DNA was then loaded in 1.2% agarose gel and run at 100V for 30 min. The WGA kit generates a pool of amplicons with an average size of 500 bp amplified products.

Nanodrop spectrophotometer (Delaware, USA) was used to measure the amount of amplified DNA. The final concentration of amplified products was 800 to 1,100 ng/µl. The ratio of OD_{260}/OD_{280} was determined to assess the quality or purity of DNA.

KaryoLite™ BACs on Beads™

Amplified DNAs were labeled with biotin-dNTP. NucleoFast 96 purification plate (Macherey-Nagel, Germany) and vacuum manifold pumps were used to purify labeled DNA. The hybridization plate was transferred into TriNest and shaken at 52ºC, 800 rpm for 5 min. The plate was denatured for 5 min at 85ºC by 9800 Fast thermal cycler (Life Technologies, USA). Biotin-labeled samples were hybridized to the beads in TriNest shaking incubator at 52ºC, 800 rpm. Post hybridization was performed, and the reporter mix was added to each plate well. Streptavidin-phycoerythrin quantifies the molecular capture interaction that occurs at the microsphere surface. The plate was loaded into the Luminex 200 instrument system and analyzed by xPonent 3.1 software (Luminex, USA). The results were interpreted following the interpretation guidelines provided by the manufacturer.

Array comparative genomic hybridization (aCGH)

Sample and control DNA was labeled with Cy3 and Cy5 fluorophores. Labeled DNA was re-suspended in the dexsulphate hybridization buffer and hybridized under cover slides. After a series of washing, the hybridized 24sure slides (BlueGnome: Illumina, UK) were scanned at 10 µm using a laser scanner (Agilent, USA). The data was analyzed using the BlueFuse software (BlueGnome: Illumina, UK) for whole chromosome loss or gain.

Statistical Analysis

The concordance of aCGH and KL-BoBs was analyzed using Microsoft Excel to compare the results from two techniques and calculate the consistency percentage of each chromosome.

RESULTS

Validation of whole genome amplification

From 45 AF cell samples tested, 40 samples could be amplified using the WGA kit. Therefore, the efficiency of WGA was 88.89%. The cause of unamplifiable five samples may be insufficient DNA quantity, cell degradation, and human error. Validation of KL-BoBs was performed in 21 AF cell samples with known chromosome abnormalities: trisomy 18 (n = 7), trisomy 21 (n = 7), and triple X (n = 7). The results of the KL-BoBs assay were 100% concordant with conventional karyotyping.

In embryo specimen samples with known chromosomal status by florescence *in situ* hybridization (FISH) assay were blindly tested with KL-BoBs. They were blastomere (triploidy) and blastocyst (normal) (Figure 1). The result did not show any differences between the profiles. Therefore, KL-BoBs cannot be used to distinguish between polyploidy and normal karyotypes.

a

Probe	/F	/M
01C1;ptel	1.02	0.97
01C2;pcen	1.12	1.08
01C3;qcen	0.95	0.93
01C4;qtel	0.94	0.96
02C1;ptel	1.05	1.01
02C2;pcen	0.97	1.02
02C3;qcen	1.05	1.01
02C4;qtel	0.95	1.01
03C1;ptel	0.97	0.97
03C2;pcen	0.85	0.90
03C3;qcen	1.00	0.99
03C4;qtel	1.09	1.04
04C1;ptel	1.06	1.02
04C2;pcen	0.98	0.94
04C3;qcen	0.88	0.91
04C4;qtel	1.02	0.96
05C1;ptel	0.92	0.93
05C2;pcen	1.08	1.04
05C3;qcen	1.11	1.01
05C4;qtel	1.11	1.07
06C1;ptel	1.08	1.05
06C2;pcen	0.85	0.91
06C3;qcen	1.08	1.01
06C4;qtel	0.95	0.96
07C1;ptel	0.98	1.01
07C2;pcen	1.00	0.96
07C3;qcen	0.95	0.93
07C4;qtel	0.97	1.01
08C1;ptel	0.97	0.96
08C2;pcen	0.91	0.90
08C3;qcen	0.98	1.01
08C4;qtel	1.08	1.02
09C1;ptel	1.00	0.97
09C2;pcen	1.05	0.97
09C3;qcen	0.94	0.99
09C4;qtel	1.02	0.94
10C1;ptel	0.91	0.88
10C2;pcen	1.02	0.96
10C3;qcen	0.97	0.99
10C4;qtel	1.09	1.07
11C1;ptel	1.12	1.11
11C2;pcen	1.00	0.99
11C3;qcen	1.14	1.10
11C4;qtel	0.88	0.91
12C1;ptel	1.06	1.02
12C2;pcen	1.18	1.10
12C3;qcen	0.97	0.99
12C4;qtel	0.86	0.91
13C1;qcen	1.02	1.02
13C2;qmid	1.03	1.04
13C3;qtel	0.95	0.93
14C1;qcen	0.83	0.83
14C2;qmid	1.05	1.02
14C3;qtel	1.09	1.08
15C1;qcen	1.14	1.05
15C2;qmid	0.97	0.94
15C3;qtel	1.05	1.01
16C1;ptel	1.08	1.02
16C2;pcen	1.14	1.08
16C3;qcen	1.08	1.05
16C4;qtel	1.08	1.04
17C1;ptel	1.02	0.94
17C2;pcen	1.05	0.99
17C3;qcen	1.17	1.10
17C4;qtel	1.02	0.96
18C1;ptel	1.02	1.04
18C2;pcen	0.88	0.96
18C3;qcen	0.85	0.87
18C4;qtel	0.95	0.94
19C1;ptel	1.00	0.96
19C2;qcen	1.05	1.02
19C3;qtel	0.95	0.94
20C1;ptel	1.12	1.08
20C2;pcen	0.97	0.96
20C3;qcen	1.06	1.11
20C4;qtel	1.00	1.01
21C1;qcen	0.98	0.97
21C2;qmid	1.09	1.11
21C3;qtel	1.06	1.05
22C1;qcen	0.98	0.96
22C2;qmid	1.09	1.02
22C3;qtel	1.00	0.93
Auto1;	1.02	1.01
Auto2;	0.98	0.99
Auto3;	0.97	0.97
X1;ptel	0.95	1.19
X2;pcen	1.02	1.26
X3;qcen	0.95	1.18
X4;qtel	0.88	1.10
Y1;ptel	0.74	0.54
Y2;pcen	0.91	0.25
Y3;qcen	0.79	0.69
Y4;qtel	0.68	0.42

b

Probe	/F	/M
01C1;ptel	0.97	0.93
01C2;pcen	1.10	1.07
01C3;qcen	1.13	1.10
01C4;qtel	0.95	0.95
02C1;ptel	0.93	0.90
02C2;pcen	1.08	1.14
02C3;qcen	1.13	1.08
02C4;qtel	0.98	1.05
03C1;ptel	0.95	0.97
03C2;pcen	0.82	0.85
03C3;qcen	0.97	0.95
03C4;qtel	1.05	1.00
04C1;ptel	0.95	0.93
04C2;pcen	1.00	0.95
04C3;qcen	0.87	0.90
04C4;qtel	1.08	1.03
05C1;ptel	0.82	0.83
05C2;pcen	1.00	0.97
05C3;qcen	1.07	0.97
05C4;qtel	1.07	1.03
06C1;ptel	1.05	1.02
06C2;pcen	0.93	1.00
06C3;qcen	1.02	0.97
06C4;qtel	0.93	0.93
07C1;ptel	0.97	0.98
07C2;pcen	1.02	0.98
07C3;qcen	0.98	0.95
07C4;qtel	0.90	0.93
08C1;ptel	1.00	0.98
08C2;pcen	0.95	0.95
08C3;qcen	0.97	0.98
08C4;qtel	1.05	1.00
09C1;ptel	1.08	1.05
09C2;pcen	1.10	1.03
09C3;qcen	0.87	0.92
09C4;qtel	1.13	1.05
10C1;ptel	0.90	0.88
10C2;pcen	1.07	1.02
10C3;qcen	0.97	0.98
10C4;qtel	1.20	1.17
11C1;ptel	1.13	1.12
11C2;pcen	0.89	0.88
11C3;qcen	1.15	1.12
11C4;qtel	0.89	0.92
12C1;ptel	1.07	1.03
12C2;pcen	0.97	0.92
12C3;qcen	0.95	0.98
12C4;qtel	0.87	0.90
13C1;qcen	1.03	1.03
13C2;qmid	0.93	0.93
13C3;qtel	1.05	1.02
14C1;qcen	0.89	0.88
14C2;qmid	0.93	0.92
14C3;qtel	1.05	1.05
15C1;qcen	1.11	1.03
15C2;qmid	1.07	1.05
15C3;qtel	1.02	0.98
16C1;ptel	1.07	1.00
16C2;pcen	1.08	1.03
16C3;qcen	0.98	0.95
16C4;qtel	1.11	1.08
17C1;ptel	0.98	0.93
17C2;pcen	0.95	0.90
17C3;qcen	1.13	1.07
17C4;qtel	1.05	0.98
18C1;ptel	1.00	1.02
18C2;pcen	0.93	1.02
18C3;qcen	1.00	1.02
18C4;qtel	0.95	0.93
19C1;ptel	1.03	1.00
19C2;qcen	1.16	1.14
19C3;qtel	1.10	1.08
20C1;ptel	1.13	1.10
20C2;pcen	0.92	0.90
20C3;qcen	1.00	1.05
20C4;qtel	1.07	1.07
21C1;qcen	0.98	0.97
21C2;qmid	1.00	1.02
21C3;qtel	1.11	1.08
22C1;qcen	0.89	0.86
22C2;qmid	1.08	1.00
22C3;qtel	1.16	1.08
Auto1;	0.98	0.98
Auto2;	1.07	1.07
Auto3;	0.95	0.95
X1;ptel	0.95	1.20
X2;pcen	1.00	1.24
X3;qcen	0.95	1.17
X4;qtel	0.89	1.10
Y1;ptel	0.77	0.56
Y2;pcen	0.79	0.20
Y3;qcen	0.80	0.71
Y4;qtel	0.79	0.47

Figure 1 Representative KL-BoBsTM results of (a) blastomere with triploidy (69,XXX) and (b) blastocyst of normal female (46,XX). Blue dots and lines represent the proportion of tested DNA compared with male reference DNA. Red dots and lines represent the proportion of tested DNA compared with female reference DNA. Green lines represent the normal signal range. The result in (a) seems to be normal, reflecting the failure of KL-BoBs to distinguish triploidy from normal karyotype.

Biopsied ICM and TE from abnormal blastocysts

The efficiency of whole genome amplification in biopsied ICM and TE was 92.3% (36/39 samples). Three samples were not amplified because of poor DNA quality and quantity. The DNA fragments' size and concentration ranged from 300 to 500 bp and 800 to 1,100 ng/µl, respectively, which were acceptable for KL-BoBs techniques. The detection of chromosomal abnormality in a total of 50 samples (11 biopsied ICM, and 39 biopsied TE) by aCGH and KL-BoBs revealed that five samples had consistent results in ICM, TE1, TE2 of KL-BoBs and TE of aCGH. Two samples had consistent results in TE1, TE2 of KL-BoBs, and TE of aCGH. One had consistent results in ICM, TE2 of KL-BoBs, and TE of aCGH. However, results from six samples were inconsistent between KL-BoBs and aCGH techniques because of mosaicism in ICM and TE. aCGH could not detect some chromosomal abnormalities (Table 1).

Table 1. Chromosomal abnormality in biopsied ICM and TE samples from the blastocysts using aCGH and KL-BoBs techniques (n=14).

Blastocyst	aCGH	KL-BoBs		
No.	**TE**	**ICM**	**TE1**	**TE2**
1	M, +22	-	M, +22	M, +22
2	F, +9, +16	-	F, +9, +16	F, +9, +16
3	F, +16	F, +16	-	F, +16
4	M, -4, +18	M, -4, +18	M, -4, +18	M, -4, +18
5	F, +21	F, -4, +21	-	F, -4, +21
6	M, +12, +X	M, -3, +9, -11, +12, +X	M, -3, -11, +X	M, -3, -11, +12, +X
7	M, -13, -18, +3, +5, +6, +9, +12, +14, +15, +16, +19	-	M, -5, -17	M, -5, -17, +21
8	F, +16	F, +4, +16	-	F, +4, +16
9	F, -15	F, -14, -15	F, +3, -14, -15	F, Normal
10	F, -15	F, -15	F, -15	F, -15
11	F, +21	F, +21	F, +21	F, +21
12	F, +7	F, +7	F, +7	F, +7
13	F, -21	F, -21	F, -21	F, -21
14	F, +X	F, +X	F, +X	F, +6, +X

aCGH: array comparative genomic hybridization, KL-BoBs: KaryoLite™ BACs on Beads™, TE: Trophectoderm, ICM: Inner cell mass, TE1: Trophectoderm 1, TE2: Trophectoderm 2, F: Female, M: Male, -: No result

Result comparison of the aCGH and KL-BoBs in blastocyst No. 9 demonstrated that both techniques provided inconsistent chromosomal abnormalities in the TE of aCGH (-15, XX). While KL-BoBs results were -14, -15, XX (ICM), +3, -14, -15, XX (TE1) and 46, XX (TE2) (Figure 2 and 3). We further analyzed 14 aneuploid blastocysts and found that 42.86 % had a single chromosome abnormality, and 57.14 % had multiple or complex chromosomal abnormalities. In biopsied ICM and TE tested by the KL-BoBs technique, single chromosome aberration was frequently present in chromosome 7, 15, 16, 21, and 22 (Figure 4). Abnormality in chromosome 21 (14.76% by KL-BoBs) was the most frequent (Figure 5). The consistency of aCGH and KL-BoBs techniques in biopsied TE showed that the average consistency was 93.8%. Chromosome 1, 2, 7, 8, 10, 20, 22 and Y were 100% consistency. Chromosome 3 was minimally consistent (79.22%) (Figure 6). Furthermore, the consistent results of biopsied ICM and TE using KL-BoBs was 98.2% average consistent. The consistent results of biopsied TE1 and TE2 samples using KL-BoBs was 97.73% average consistent.

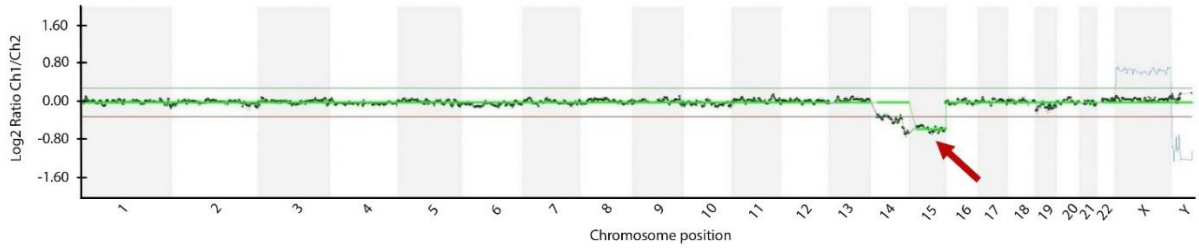

Figure 2 aCGH result of biopsied TE from blastocyst No. 9 (-15, XX). The chromosome numbers are shown in the X-axis and the arrow indicates an abnormal region (chromosome 15).

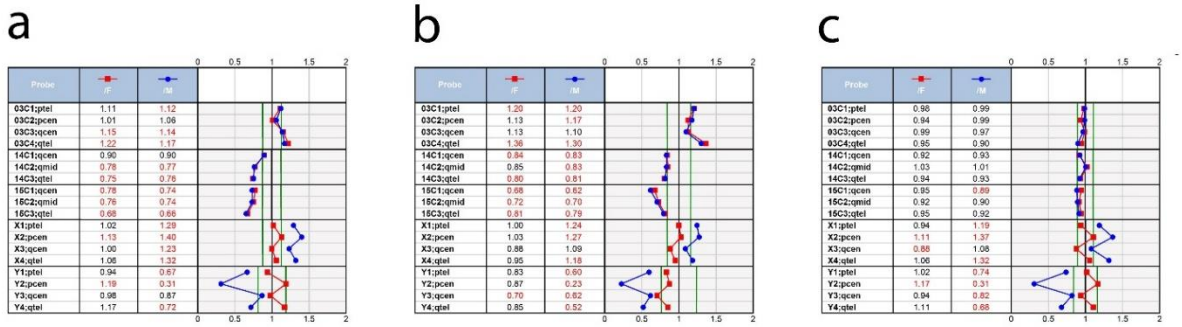

Figure 3 KL-BoBs^{TM} results from blastocyst No.9: (a) biopsied ICM (-14, -15, XX), (b) biopsied TE1 (+3, -14, -15, XX) and (c) biopsied TE2 (46,XX). Blue dots and lines represent the proportion of tested DNA compared with male reference DNA. Red dots and lines represent the proportion of tested DNA compared with female reference DNA. Green lines represent the normal signal range.

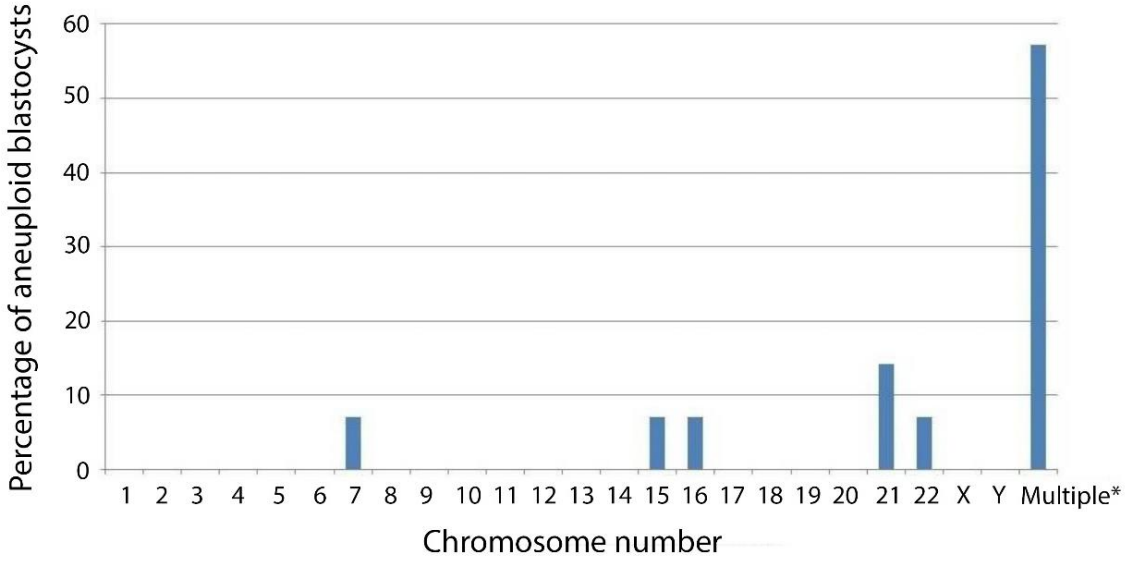

Figure 4 Number of aneuploid blastocysts with a single abnormal chromosome and multiple abnormal chromosomes; Multiple* = The presence of more than one chromosomal aneuploidy. The chromosome numbers and the percentage of aneuploid blastocysts are shown in the X- and Y-axis, respectively.

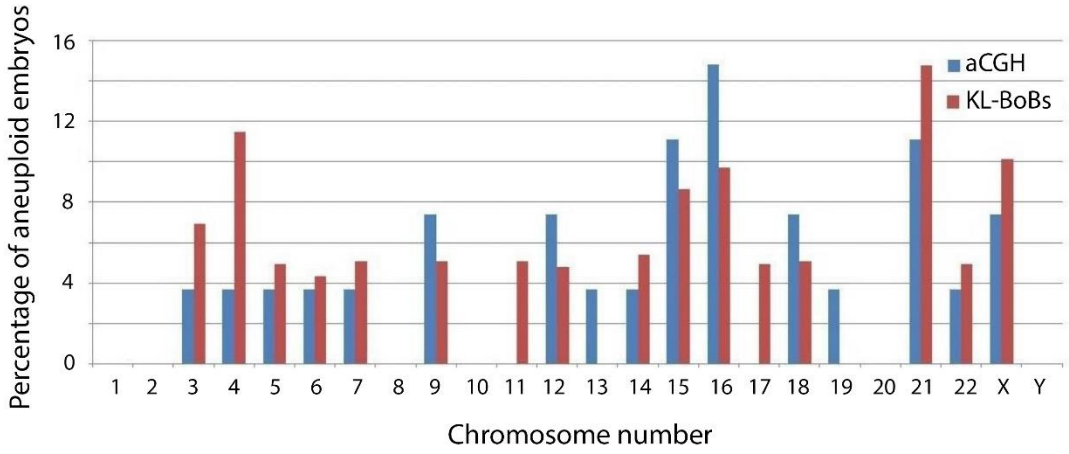

Figure 5 The proportions of aneuploid embryos with combined single and multiple chromosomal abnormalities in KL-BoBs™ and aCGH techniques. The chromosome numbers and the percentage of aneuploid embryos are shown in the X- and Y-axis, respectively.

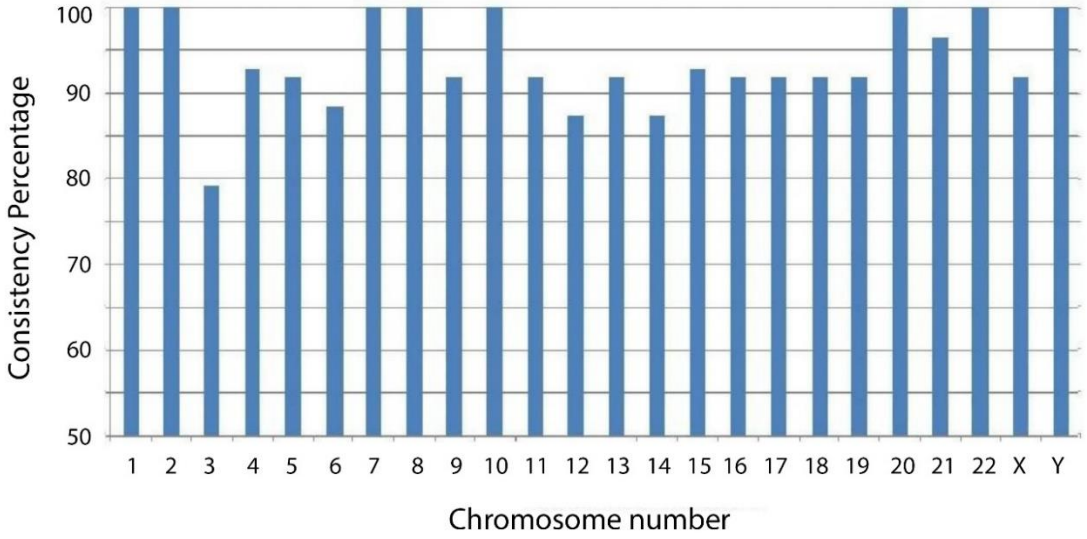

Figure 6 Comparison of the consistency percentage of aCGH and KL-BoBs™ techniques in biopsied TE. The chromosome numbers and the consistency percentage are shown in the X- and Y-axis, respectively.

DISCUSSION

We aimed to evaluate the efficiency of WGA and KL-BoBs as a routine diagnostic assay in a clinical setting. We found that the amplification rate was approximately 89%, but the amplification efficiency of at least 90% for each marker is recommended (Harton *et al.* 2010). The cause of amplification failure may be due to human error and inappropriate source of DNA that was unsuitable for further analysis. When comparing KL-BoBs with karyotyping method, the results were 100% concordant for trisomy 18, trisomy 21, and triple X in amniotic fluid cell samples. The accurate detection of KL-BoBs depended on the coefficient of variation <8 % to detect loss and gain of chromosome regions as recommended by the manufacturer' s analysis guidelines. KL-BoBs assay was limited by its inability to detect polyploidy and structural abnormalities. Therefore, blastomere with a 69,XXX by FISH were identified as normal females by KL-BoBs.

In the present study, embryo biopsy was done in duplicate, and the second biopsy was performed in TE and ICM of abnormal blastocysts in which the first biopsy and aCGH had been completed. The amplification rate was ~92%. Three samples were not

selected to detect chromosome abnormalities by KL-BoBs because of deficient quality of the trophectoderm cells. Chromosomal abnormality detection was subjected to KL- BoBs assay, and the results demonstrated that most of the blastocysts had multiple chromosomal abnormalities (57.14 %). However, previous studies reported that single chromosomal abnormality (62.3%) occurred more often than multiple chromosomal abnormalities (37.7%) (Liu *et al.* 2012). In this study, chromosome 21 was the most frequent single chromosomal abnormality (14.76%), which was concordance to previous studies (11.3%) (Liu *et al.* 2012). Our data showed that the average consistent result of aCGH and KL-BoBs technique was 93.8%. Therefore, trophectoderm cells which had different cell groups were used to detect by these two techniques. Six cases showed discordant results between KL- BoBs and aCGH due to chromosome mosaicism and gain or loss of DNA below threshold value used to identify gains of losses in aCGH analyses. The average consistent of biopsied ICM and TE using KL- BoBs was 98.2%, and the average consistent of biopsied TE1 and TE2 was 97.73%.

KL- BoBs is potentially very useful as the first-line test for screening chromosomal abnormality in PGT-A. A prenatal diagnosis should be performed to confirm chromosome abnormality in the fetus. The advantages of KL-BoBs are relatively easy laboratory techniques. These advantages include: (i) easy for interpretation, (ii) low cost, (iii) good efficiency, (iv) require small amount of input DNA (~50–240 ng), (v) DNA storage availability, (vi) the robust nature of the assay, (vii) low failure rate and (viii) high throughput of up to 92 samples per run. Moreover, KL-BoBs can be performed within 24 hours. However, it may not have a significant edge over aCGH as a rapid diagnostic test. The aCGH and NGS analysis are more comprehensive compared with KL-BoBs, where the resolution is lower and consequently result from interpretation is less complicated. Moreover, aCGH and NGS are a high cost, and the patients may not be affordable in many countries.

Although the data were obtained from a limited number of embryos (n = 14) and patients (n = 3), to our knowledge, the present study is the first research to use KL-BoBs to report these preliminary results for preimplantation genetic screening. Besides, the limitation of this study was WGA products from different groups of TE, which was used to compare aCGH and KL-BoBs techniques. It could provide an inconsistent result because of mosaicism. Further studies remain necessary to use more embryos in different patient populations to detect normal chromosomes in ICM and the same WGA product should be used to compare aCGH and KL- BoBs techniques to provide more reliable results.

In conclusion, our results demonstrated that the results from KL-BoBs had concordance with aCGH technique to detect chromosomal abnormalities for preimplantation genetic testing for aneuploidy in abnormal blastocysts. Consistency of biopsied ICM and TE was 98.2%, which indicated that biopsied TE in human blastocysts could predict chromosome information in ICM. Because of the ethical issue, therefore, normal chromosomes in ICM were not detected in this study.

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