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ORIGINAL ARTICLE

Validation of Next-Generation Sequencer for 24-Chromosome Aneuploidy Screening in Human Embryos

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Background: Next-Generation Sequencing (NGS) is the latest approach for preimplantation genetic diagnoses (PGD).
Aim: The purpose of this study was to standardize and validate an NGS method for comprehensive chromosome screening and to investigate its applicability to PGD.
Methods: Embryo biopsy, whole-genome amplification, array comparative genomic hybridization (aCGH), and semiconductor sequencing were employed.
Results: A total of 204 whole-genome-amplified products were tested with an NGS-based protocol, from which 100 samples were used for standardization and to evaluate the quality of the results produced by this new technique. The remaining 104 samples tested by NGS were previously analyzed by using the aCGH protocol to determine the sensitivity and specificity of this new technique. In total, 4896 chromosomes were assessed, out of which 196 carried a copy number imbalance. NGS sensitivity and specificity for calling aneuploidy was 100%.
Conclusion: This is the first study reporting preclinical validation and accuracy assessment of the Ion Torrent Personal Genome Machine (PGM) NGS-based comprehensive chromosome screening method using blastomeres and blastocysts. The NGS proved to be a robust methodology and is ready for clinical application in reproductive medicine, with the major advantage of low cost and enhanced precision when compared with other technologies used for comprehensive chromosome screening.

Keywords: comprehensive chromosome screening, preimplantation genetic diagnosis, array comparative genomic hybridization, Next-Generation Sequencing

Introduction

Preimplantation genetic diagnosis (PGD) has transformed the approaches to infertility patients in the IVF settings. PGD was proposed as a method to improve Assisted Reproductive Technology (ART) outcomes by distinguishing the chromosomally normal embryos from those with potentially lethal forms of chromosome aneuploidy (Munne *et al.*, 1993); it also aims at increasing implantation and ongoing pregnancy rate for IVF patients, reducing the time to pregnancy, lowering the incidence of miscarriage, and reducing the risk of aneuploidy condition at term (Wilton, 2002). Chromosomal aneuploidy is recognized to be a significant contributing factor in implantation failure and spontaneous miscarriages (Lathi *et al.*, 2008), and it is likely to be responsible for most IVF failures. Aneuploidies are common in early human embryos (Harper *et al.*, 1995; Munne and Cohen, 1998). Trisomic and monosomic embryos account for ≥10% of human pregnancies and for women nearing the end of their reproductive life span, this incidence may exceed 50% (Nagaoka *et al.*, 2012). Aneuploidy rates are higher in oocytes and embryos from women of advanced maternal age (Hassold *et al.*, 1980), probably due to meiotic recombination defects exacerbated by age (Lamb *et al.*, 1996). Therefore, embryonic aneuploidy is likely to be the main factor responsible for the concomitant decrease in implantation rates with advanced maternal age.

The initial studies of PGD involved the biopsy of a single blastomere from cleavage-stage embryos, and the fluorescence *in situ* hybridization (FISH) technique was used to detect chromosomal aneuploidies. However, the clinical results were disappointing. One possible reason for the poor clinical performance has been attributed to the well-known limitation of the FISH technique, which screens only minority of chromosomes, which are mostly implicated in spontaneous miscarriages and live births with chromosomal abnormalities (Rubio *et al.*, 2013), which are not necessarily the most relevant in early embryos. This may lead to the transfer of reproductively incompetent embryos, thus reducing the diagnostic accuracy of FISH technology (Harper

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et al., 2010; Harton *et al.*, 2013). Therefore, the focus has now been shifted to different technologies that allow for comprehensive screening of chromosomes or full karyotyping, to provide a more accurate assessment of the reproductive potential of embryos.

Currently, several approaches toward 24-chromosome analysis have been developed under the general denomination of comprehensive chromosome screening. This PGD method includes array comparative genomic hybridization (aCGH) (Colls *et al.*, 2007), metaphase comparative genomic hybridization (Fragouli *et al.*, 2006), single-nucleotide polymorphism microarray (Gutierrez-Mateo *et al.*, 2011), and quantitative polymerase chain reaction (Yang *et al.*, 2012). aCGH was the first technology to be widely available for the 24-chromosome copy number analysis (Wells *et al.*, 1999; Wilton *et al.*, 2001). This technique involves the labeling and the competitive hybridization of differentially labeled test and reference DNA samples. Each probe is specific to a different chromosome region and occupies a discrete spot on the microarray slide, and specific software is used to detect the imbalances in any of the 24 chromosomes. Multiple randomized clinical trials (Yang *et al.*, 2012; Forman *et al.*, 2013; Rubio *et al.*, 2013) have shown significantly improved consistency and predictive value for aneuploidy assessment, as well as high implantation and pregnancy rates when aCGH as the comprehensive chromosome screening method was used (Wells *et al.*, 2008; Fiorentino *et al.*, 2011; Gutierrez-Mateo *et al.*, 2011).

Next-Generation Sequencing (NGS) is the newest technology to be incorporated into PGD. Its rapid development has generated an increasing interest in determining whether NGS could be reliably applied to comprehensive chromosome screening or this technique is in advance to the current comprehensive chromosome aneuploidy screening technologies. The principle of NGS involves massive parallel sequencing of small DNA fragments until a sufficient sequencing depth (number of sequence reads covering a given position in the genome) is achieved. The sequencing data from chromosomes across the genome are then compared with a reference genome and analyzed for whole chromosome aneuploidy and for segmental chromosome imbalance by using specific software (Yin *et al.*, 2013). Compared with other PGD methods, NGS has an advantage that it could potentially test both aneuploidy and monogenic diseases simultaneously. In contrast, the other PGD techniques only detect chromosome abnormalities (Colls *et al.*, 2007).

However, new diagnostic technologies need thorough validation to determine its preclinical specificity and sensitivity. Such studies should be compared with an established method before they might be considered within the actual medical context. The present validation study investigates the accuracy of an NGS methodology for comprehensive chromosome screening as a step toward its application in the diagnosis of chromosome aneuploidy on embryos at cleavage stage or blastocyst stage.

Materials and Methods

Experiment design

This study was carried out in two steps. The first step was to standardize the sequencing protocol for 204 samples as per Ion Reproseq PGS kit. The second step was to validate the specificity and sensitivity of this new technique.

Consent approval

Samples obtained in this study were obtained with patient consent.

Standardization and whole-genome amplification

The 204 whole-genome-amplified products (SurePlex DNA amplification system from Illumina) were quantified with Qubit dsDNA HS (high sensitivity) assay kit as per the manufacturer's manual (Invitrogen). The average DNA concentration of the samples was between 50 and 70 ng/μL. These amplified WGA products (SurePlex DNA amplification system from Illumina) were processed by NGS (Ion Reproseq PGS kit). The barcodes were assigned as per Ion Reproseq PGS kit protocol (ThermoFisher Scientific).

Array comparative genomic hybridization

aCGH was performed as per current 24Sure V3 protocol (Illumina).

Library pooling, purification, and quantification

Barcoded whole genome amplification (WGA) products were pooled, purified, quantified, and processed by following the Thermo Fisher Scientific Ion Reproseq PGS library preparation kit protocol. The barcoded samples libraries were pooled in 24 sample multiplexes. The sequencing run was performed by using the Ion PGM Sequencing 500 Kit v2, and samples were loaded on to Ion 318 Chip Kit v2.

Sequencing analysis

The data generated from Torrent server 5.0.4 were analyzed by using software version 5.0.4 for reads filtering, base calling, barcode filtering, and alignment to the human genome hg19 reference. The plug-in software was also used to check the quality of experiment for each sample with respect to a sufficient number of reads and chip loading percentage. For data analysis, the samples were processed through the Ion Reporter Software version 5.0 by using ReproSeq low-pass whole-genome aneuploidy workflow that can detect aneuploidies and large chromosome abnormalities from a single whole-genome sample with low coverage (minimum 0.01×). Normalization was performed by using an informatics baseline generated from multiple normal samples. The analysis for aneuploidy detection was performed by using an algorithm based on a hidden Markov model. The algorithm uses read coverage across the genome to predict the copy-number or whole-number ploidy status (i.e., 0, 1, 2, 3, etc.). Before copy-number determination, read coverage is corrected for guanine-cytosine (GC) bias and compared with a preconfigured baseline obtained from 10 normal male samples that were processed in a manner representative of the method described herein. The use of 10 samples for baseline calculation substantially reduces the sample-to-sample variance in coverage and results in a smaller number of false-positive calls. The analysis visualization can be viewed in integrated genome viewer (IGV) light version 5.0, and scoring of aneuploidy was based on visualization of the IGV profile indicating losses and gains of the

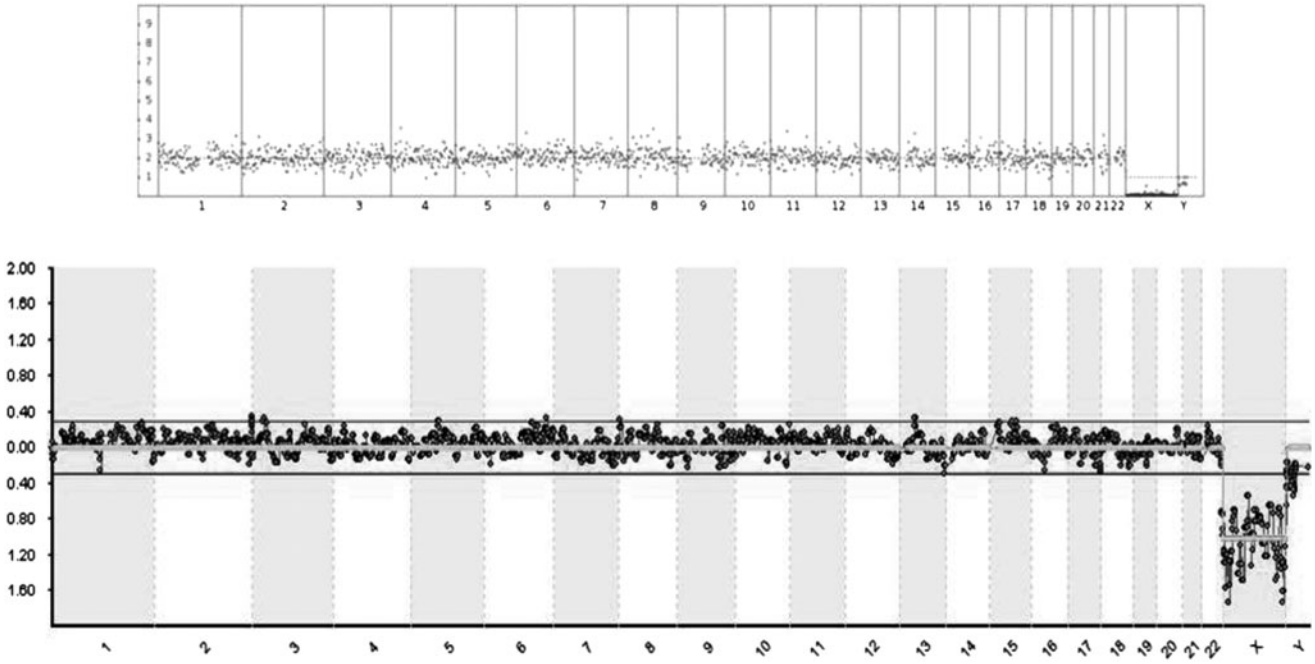

FIG. 1. The *upper panel* is the graphical visualization of the NGS analysis using IGV. The *lower panel* is the graphical representation of the array profile. Analysis of both samples is 45,Y. IGV, integrated genome viewer; NGS, Next-Generation Sequencing.

whole chromosome coupled with confidence and precision metrics. Confidence is defined as a log ratio between the observed ploidy value and the expected value. Large confidence values indicate that the algorithm is very certain that the ploidy state differs from the expected. Precision is defined as a log ratio between the likelihood of the assigned ploidy state and a next-closest state. Low precision (<10) denotes uncertainty in the absolute ploidy value assignment. It is possible that high ploidy states (e.g., copy number state >5) will have low precision and high confidence, indicating that the algorithm is uncertain about the absolute ploidy value but quite certain about the existence of a ploidy state that is different from the expected (for example, high confidence means copy number increase is present, but uncertainty in whether the exact copy number is 5 or 6).

Evaluation of sensitivity and specificity of 24-chromosome aneuploidy screening by NGS

To assess the reliability of comprehensive chromosome screening by NGS, the following criteria were used: (1) Sensitivity defines the probability that the aneuploidy call will be true when aneuploidy is present (True Positive). (2) Specificity defines the probability that the aneuploidy call is negative when aneuploidy is not present (True Negative). (3) Results were considered concordant when the original results and the NGS results were categorized in the same grouping, euploid, abnormal, and complex abnormal.

An aneuploid result was defined as a whole chromosomal loss (monosomy) or gain (trisomy). A complex abnormal profile was a combination of five or more trisomic or monosomic events.

After analysis, results were compared with those previously obtained with aCGH to determine sensitivity and specificity of this new technique.

Results

The 204 whole-genome-amplified products used in this validation process were obtained from patients undergoing PGD with indications of advanced maternal age, repetitive IVF failure, recurrent miscarriages, or severe male factor.

Using the NGS technology, successful results were obtained from 200 (98%) out of 204 samples. No results were obtained for four samples (2%) due to degraded DNA. Among 204 samples, 100 WGA products were assessed blindly (using NGS protocol only) and the remaining 104 samples that were previously diagnosed with aCGH were assessed by NGS protocol, to check the consistency of NGS for aneuploidy screening.

The 204 samples were tested in a batch (24 samples on Ion 318 Chip Kit V2) after being barcoded (Ion Single Seq Barcode Set 1), with an average of 70–80% of loading density. The average number of reads obtained depends on the number of samples loaded per chip per run. A 24-sample multiplex will produce an average of 123,660 filtered reads per sample. NGS and aCGH results are shown in Figure 1.

One hundred samples were blindly assessed by using NGS protocol only. Out of these, 4 (4%) showed a chaotic profile due to the presence of degraded DNA, 18 (18%) were

Table 1. Next-Generation Sequencing and Array Comparative Genomic Hybridization Concordance Analysis

	Normal	*Abnormal*
Concordant	47	56
Discordant	1	0
Total	48	56
Concordance (%)	98	100

Table 2. Discrepancies Between Next-Generation Sequencing and Array Comparative Genomic Hybridization Results of Same Embryos

Sample	*NGS results*	*aCGH results*	*Comment*
122	46,XX, +5, −7, −16, +22	46,XX, −16, +22	Abnormal
124	45,XY, +15, −16, −18	46,XY, +15, −16	Abnormal
137	48,XY, +6, +16, +20, −21	45,XY, −9, +16, −21	Abnormal
139	44,XX, −7, −21	42,XX, −7, −8, −13, −21	Abnormal
142	44,XX, −12, −22	45,XX, −22	Abnormal
143	47,XX, +19	48,XX, +15, +19	Abnormal
152	45,XX, −22	44,XX, −2, +12, −15, −22	Abnormal
153	45,XX, −22	45,XX, −2, +12, −22	Abnormal
157	46,XX, +11, −14, +15, −22	46,XX, −2,+11, +12, −14, +15, −22	Abnormal

NGS, Next-Generation Sequencing.

euploid, 9 (9%) had sex chromosome aneuploidies, 68 (68%) showed autosomal aneuploidies, and 1 (1%) had both autosomal and sex chromosome aneuploidies.

NGS results of the remaining 104 samples were compared with the previously established aCGH diagnosis. As per aCGH results of 104 samples, 48 samples were euploid and 56 samples were recorded as aneuploid.

On comparison of NGS and aCGH results, there was 98% (47/48) concordance rate between euploid embryos and 100% (56/56) for aneuploid embryos (Table 1). Therefore, NGS specificity and sensitivity for aneuploidy screening at embryo level is 100%. From 104 WGA products, a total of 2496 chromosomes were assessed; of these, 2480 produced concordant results and 15 produced discordant results (missed or extra chromosome calls). However, nine samples classified as abnormal by aCGH showed some discrepancies in the aneuploidies detected when analyzed by NGS, but other abnormalities within the same embryo were confirmed and, therefore, the embryos were still classified as abnormal (Table 2).

Discussion

NGS is an emerging technology that provides high-throughput and high-resolution sequencing data for chromosome analysis; however, it has yet to be validated for PGD application where the samples used are single cells.

We performed a preclinical validation study of NGS-based 24-chromosome aneuploidy screening protocol, on embryo biopsies in which diagnosis had previously been established by the well-established aCGH method.

In this study, the technical accuracy of NGS was tested in two phases. The first phase involved the standardization of this new technique for performing comprehensive chromosome screening, where the results demonstrated 96% of success rate. The second phase involved the assessment of WGA products through NGS. These WGA products were selected from previously performed clinical PGD cycles in which diagnosis was established with aCGH. The results achieved clearly proved the ability of NGS to screen for chromosome aneuploidies.

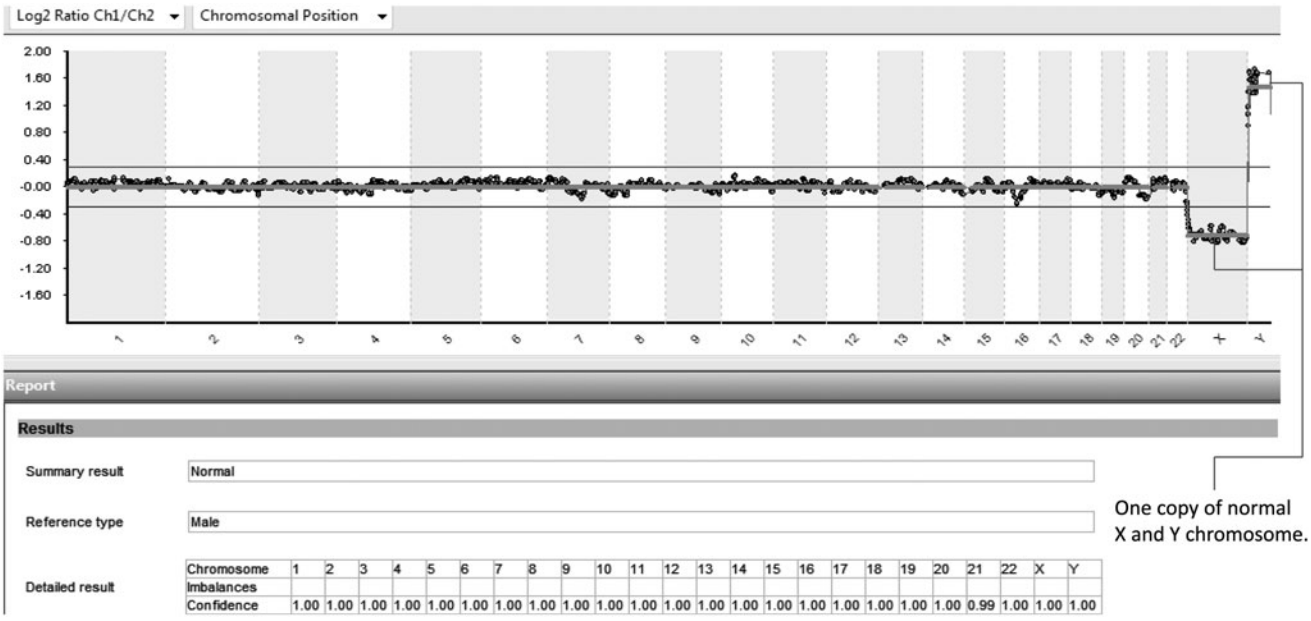

FIG. 2. The panel shows BlueFuse profile of normal male control (46,XY) produced by aCGH. aCGH, array comparative genomic hybridization.

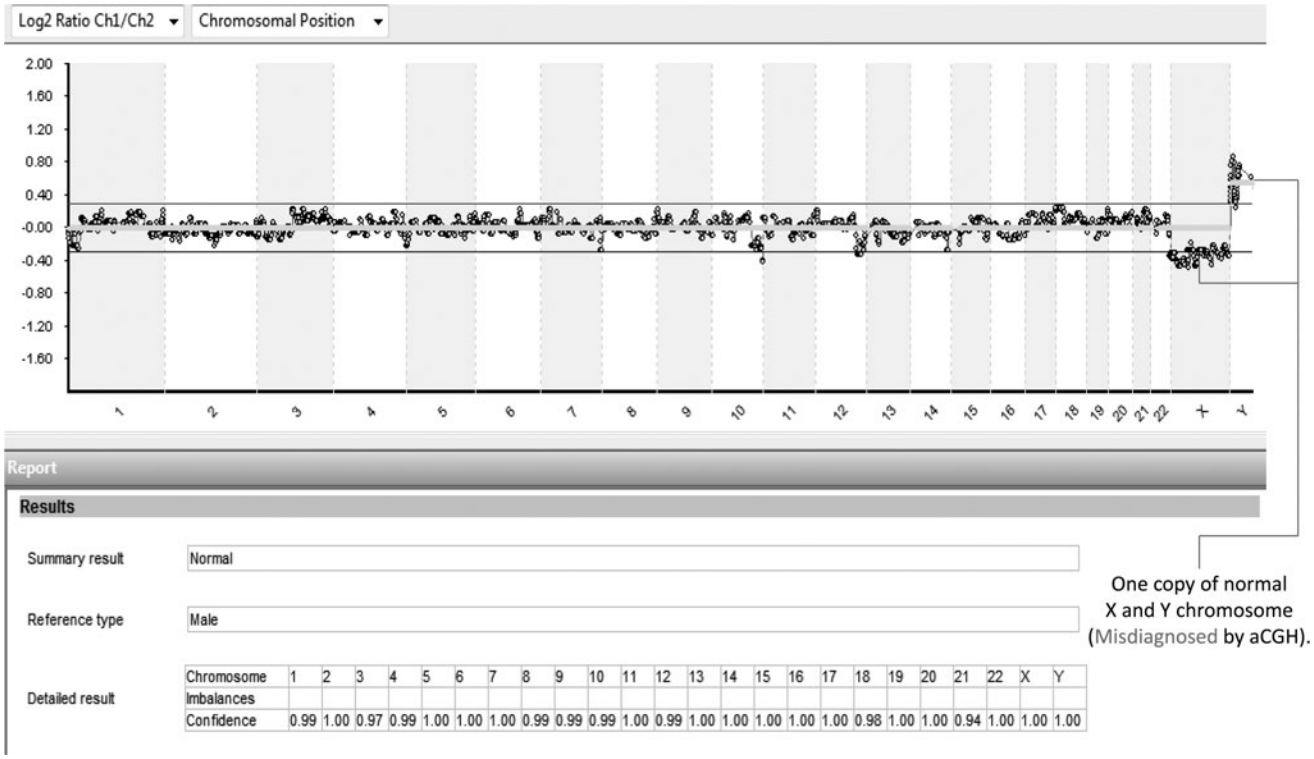

FIG. 3. The panel shows BlueFuse profile of sample misdiagnosed as normal male (46,XY) produced by aCGH.

NGS analysis of the samples in this study resulted in 98% chromosome copy number assignment consistency. Ninety-eight percent of embryos diagnosed as euploid by aCGH were confirmed as euploid by NGS, with the exception of one embryo that was 46,XY as per aCGH result and came out to be 47,XYY by NGS, as shown in Figures 2–5. The misdiagnosis of aCGH may be due to its limitation of undercall changes in noisier assays. The algorithms work by assessing the distance of a proposed change from the noise. If the noise level is high, the algorithms can sometimes miss a change. All embryos diagnosed as aneuploid by aCGH were confirmed as aneuploid by NGS (100% 24-chromosome diagnosis consistency).

However, there were nine samples in which difference was seen between results obtained by using NGS and aCGH (all biopsies taken from blastocyst stage), as seen in Table 2. These nine embryos had multiple chromosome aneuploidies, some of which are detected by both methods, whereas others were only observed by using one of the two techniques. It is typical for highly abnormal embryos to display mosaicism

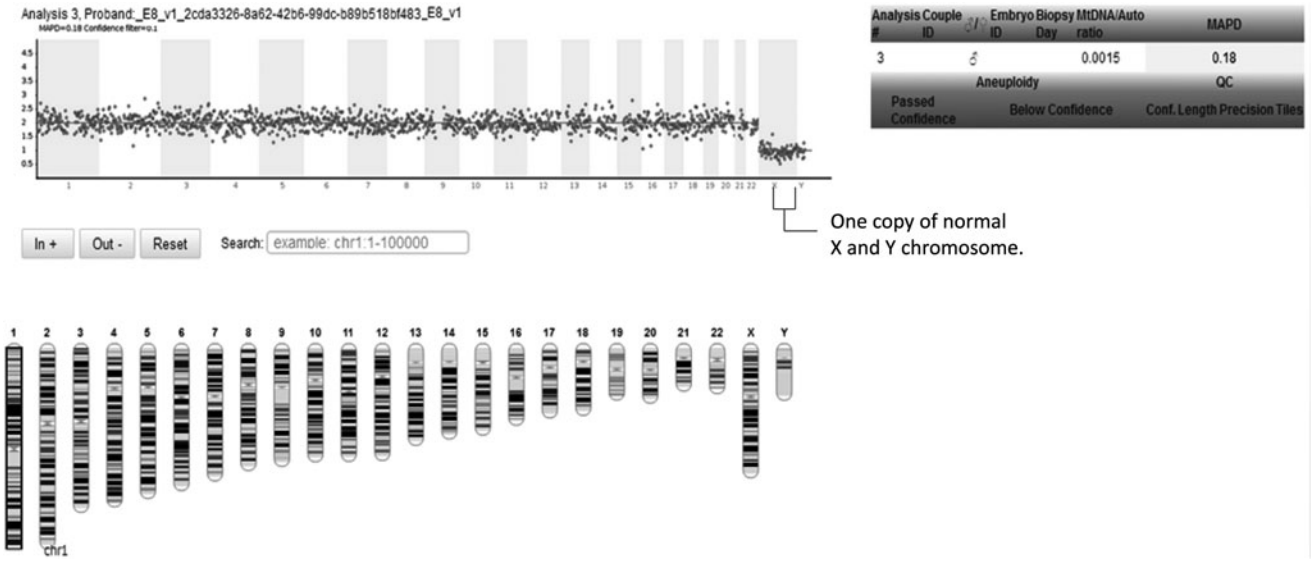

FIG. 4. The panel shows IGV profile of normal male control (46,XY) produced by NGS.

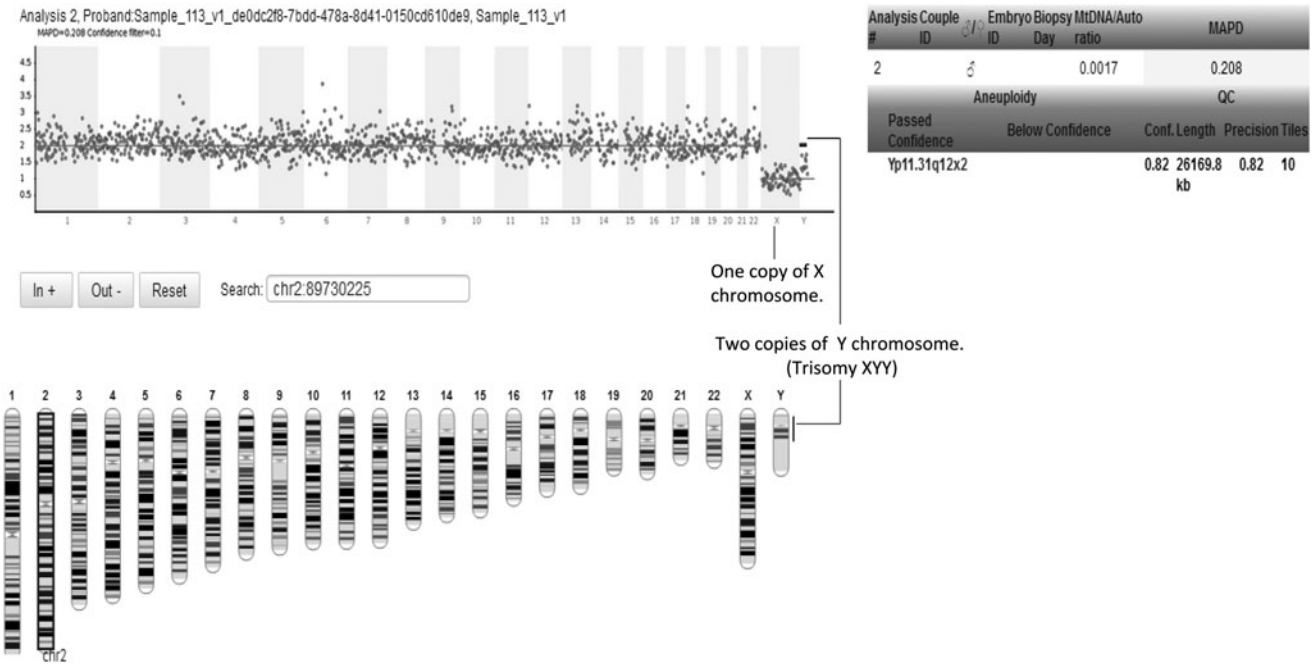

FIG. 5. The panel shows IGV profile of sample (misdiagnosed earlier with aCGH) diagnosed with trisomy of XYY produced by NGS.

(Colls *et al.*, 2007), and, consequently, some cytogenetic divergence between biopsy specimens is expected. Importantly, despite a degree of discrepancy in terms of predicting karyotype, the actual clinical diagnosis (i.e., abnormal) was the same for these embryos regardless of whether NGS or aCGH was used.

In addition to the 24-chromosome aneuploidy screening, NGS protocol presented here has also shown accurate detection of segmental changes (as small as 14 MB in size), indicating that diagnosis of partial aneuploidies is well within the ability of this technology (Yin *et al.*, 2013; Fiorentino *et al.*, 2014). It is, therefore, reasonable to assume that patients with balanced translocations will also benefit from NGS-based chromosome comprehensive screening. In addition, NGS also generate mitochondrial DNA values from the same biopsy without the need for multiple unique technologic platforms. This additional genetic information from human embryos can be useful for diagnostic and research purposes. Further advantages of NGS compared with aCGH is its ability to detect haploidy, polyploidy, cohybridization of control samples is not necessary, and finally the massive parallel sequencing of multiple samples on the same chips by using DNA barcoding methodology. These advantages of the NGS method may ultimately lead to reducing the cost per patient, allowing IVF couples a wider use of PGD for choosing competent embryos for transfer.

Although there are many advantages of this new technology, the limitations must also be considered. NGS cannot directly detect balanced chromosome rearrangements because there is no imbalance in the total DNA content. Moreover, although NGS has the potential to detect haploidy and polyploidy with the use of allele ratio, the sequence coverage of the protocol is insufficient to enable allele detection, which requires a higher read depth. It is also important to consider the cost of the NGS instrument. Potential cost benefits may not be achieved if there are insufficient samples available to fully utilize the available sequencing capacity.

To our knowledge, this is the first study of its kind reporting clinical validation and accuracy assessment of Ion Torrent PGM NGS-based comprehensive chromosome screening of single cells. Given the high degree of concordance between NGS and aCGH, NGS-based aneuploidy screening appears to be a robust methodology ready to find a place in the routine clinical application.

At present, this study indicates that NGS provides a reliable high-throughput methodology for 24-chromosome aneuploidy screening. This approach has the potential to represent a useful strategy for PGD.

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Authors' Contributions

K.S.: Study design, optimization of laboratory methods, execution of the project work, analysis of data and results, and preparation of the article; R.D.: Guidance for the laboratory work, analysis of data, and results and preparation of the article; F.A. and R.A.: Embryo biopsy and tubing and reviewing the article; B.P.: Support in the enrollment of patients in the study, clinical documentation for the patients and proforma filling, and patient referral for molecular studies and reviewing of the article.

Author Disclosure Statement

No competing financial interests exist.

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