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Detecting trisomy in products of conception from first-trimester spontaneous miscarriages by next-generation sequencing (NGS)

Jing Xu[a], Min Chen[b], Qi Yun Liu[a], Shun Qin Hu[a], Li Rui Li[a], Jia Li[a], Run Mei Ma[a,*]

Abstract

Miscarriage is the spontaneous loss of a clinically established intrauterine pregnancy before the fetus has reached viability. In order to compare the performance of traditional G banding karyotyping with next-generation sequencing (NGS) for detecting common trisomies in products of conception (POC). Chromosome abnormalities were detected by high-resolution G banding karyotyping and NGS. A total of 48 miscarriage samples, including 20 samples without karyotype result and 28 with karyotype results were selected and coded for analysis by NGS. The multiplex PCR analysis of maternal and miscarriage DNA for single nucleotide polymorphism (SNP) markers were used to simultaneously monitor maternal cell contamination (MCC), chromosomal status, and sex of the miscarriage tissue. NGS detection results of 21 chromosome abnormalities were consisted with that in karyotyping examination. These chromosome abnormalities samples included 9 chromosome 16 trisomies, 3 chromosome 22 trisomies, 2 chromosome 7 trisomies, 2 chromosome 18 trisomies, 1 chromosome 4 trisomies, one chromosome 10 trisomies, 1 chromosome 13 trisomies, 1 chromosome 15 trisomies and 1 sex chromosomal aneuploidies (45, X). Meanwhile, NGS analysis of seven chromosome normalities was adapted to the karyotyping examination. Therefore, NGS combined with multiplex PCR is an effective method to test trisomies in POC. The results mentioned above will contribute to a detailed understanding of the first-trimester spontaneous miscarriages.

Abbreviations: array-CGH = array–comparative genomic hybridization, CNVs = copy number variants, FISH = fluorescence in situ hybridization, MCC = maternal cell contamination, MLPA = multiplex ligation-dependent probe amplification, NGS = next generation sequencing, POC = products of conception, QF-PCR = Quantitative fluorescent polymerase chain reaction, SNP = single nucleotide polymorphism.

Keywords: karyotype, miscarriages, next generation sequencing (NGS), POC, trisome

1. Introduction

Miscarriage is the spontaneous loss of a clinically established intrauterine pregnancy before the fetus has reached viability. Many studies have demonstrated that 50% of fertilized eggs were dead and were spontaneously aborted, which was usually occurred before the pregnancy is recognized. Among women who know they are pregnant, the miscarriage rate is 15% to 20%, which is the most frequent complication of a pregnancy.[1–2] Around a quarter of women had experienced at least one miscarriage during their lives.[3] Half of the first-trimester miscarriages (<12 weeks gestational age) are caused by fetal chromosome abnormalities, which was often diagnosed by conventional techniques.[4–8] Currently, researchers had indicated that the risk of a fetal trisomy was increased with the rising maternal age.[9] Meanwhile, the average age of women bearing their first child had increased strongly over the last 2 decades in western countries, thus the occurrence rate of fetal trisomy was gradually elevated.[10] Based on the evidence mentioned above, miscarriage sample testing was important to determine the genetic factors that contributed to understanding the products of conception (POC).

Cytogenetic evaluation of the POC was an important approach to determine the cause of pregnancy loss. This method is helpful to estimate the recurrence risk and give advice for subsequent pregnancies. However, this approach still had many challenges to overcome. For example, the long turnaround time of cell culture and the high failure rate. Techniques such as Chromosomal Comparative Genomic Hybridization (CGH), array–Comparative Genomic Hybridization (array-CGH), Fluorescence in situ hybridization (FISH), Multiplex Ligation-dependent Probe Amplification (MLPA) and Quantitative Fluorescent Polymerase Chain reaction (QF-PCR) have overcame some disadvantages inherited from conventional cytogenetic techniques, including poor chromosome preparations, culture failure, or maternal cell contamination. Meanwhile, these molecular biological techniques offered multiple advantages, including short turnaround time and

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This study was approved by the ethics committee of the First Affiliated Hospital of Kunming Medical University, every patient of this study fully understand experimental contents and signed the informed consent.

The authors have no conflicts of interest to disclose.

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

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[a] First Affiliated Hospital of Kunming Medical University, Kunming, [b] Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, China.

[*] Correspondence: Run Mei Ma, First Affiliated Hospital of Kunming Medical University, Kunming, China (e-mail: Marunmei1234@126.com).

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high resolutions. However, it was notable that only array-CGH could screen the chromosome abnormality in the whole genome. Previous studies suggested that these molecular biological techniques have identified more abnormalities in early miscarriages.[11–13] However, these molecular technologies still had its drawbacks. For example, they showed no regard for the type of the received tissue when compare with cytogenetic evaluation.

NGS is a technology that parallel sequences massive amounts of short DNA strands from randomly fragmented copies of a genome.[14–24] Currently available NGS platforms include the Illumina HiSeq/MiSeq, Life Technologies Ion Torrent/Ion Proton, Life Technologies SOLiD, and Roche 454. In contrast to Sanger sequencing, which produces a single long (often >1 kb) read using dye terminator chemistry, NGS methods typically generate millions of short reads on the order of 50 to 300 bp using reversible sequencing chemistries.[25] NGS-based diagnostics are rapidly becoming part of the clinical genomic testing and are now routinely offered by many commercial and academic laboratories. One of the key features of NGS-based diagnostics is its ability to detect a full range of genetic variation, which offers the potential to streamline testing. Clinically, multiple approaches, including target sequencing and low-depth whole-genome sequencing, had been used in practical application. Therefore, whole-genome data is low coverage and suitable for the detection of constitutional variants in humans.

In this study, we have employed a low-depth whole-genome sequencing method to examine the trisomies in products of conception from first-trimester spontaneous miscarriages. Maternal cell contamination (MCC) was ruled out with a multiplex PCR method. Testing results of NGS combined with multiplex PCR was consisted of the traditional G banding karyotyping method. The turnaround time of NGS combined with multiplex PCR was significantly shorter than the G banding karyotyping method. The results obtained in this study provide a novel strategy to rapid test the chromosome composition in the POC sample.

2. Methods

2.1. Microdissection and DNA preparation

Women were recruited in this study with the signs of miscarriage, including lower abdominal pain, symptoms, uterine cramping, vaginal bleeding or loss of pregnancy presented at the Department of Obstetrics and Gynecology, First Affiliated Hospital of Kunming Medical University between 7 and 12 weeks gestational age for assessment and ultrasound examination. If fetal demise was confirmed by transvaginal pelvic ultrasound, the products of conceptions were retrieved from the uterus by aspiration. The tissue fragments were examined microscopically for the presence of placental chorionic villi. Regions of chorionic villi were carefully dissected from the tissue and thoroughly washed three times in PBS buffer to minimize any maternal cell contamination (MCC). Several clumps of villi were retained for karyotyping and the remainder of the villi frozen at -20°C for subsequent DNA isolation. Maternal decidua/endometrium, chorionic villi, and fetal tissue (if present) were microdissected separately from cytokeratin-stained slides with the use of a narrowly cut, thin razor blade grasped by a hemostat under a dissecting microscope. The chorionic villi were placed in 100 mL of lysis buffer containing 10 mM of tris (hydroxymethyl) aminomethane hydrochloride, 1 mM of ethylenediaminetetraacetic acid, 0.5% Tween 20 (Fluka, Milwaukee, WI), and 200 mg/mL of proteinase K (added daily) for twelve hours at 65°C with continuous agitation. The proteinase K then was denatured by heating to 95°C for 10 minutes and Genomic DNA was extracted using the General AllGen kit (Cwbiotech cw2298). The kit was used according to the manufacturers' instructions and DNA samples were stored at −20°C.

2.2. Multiplex PCR

One hundred twenty SNP sites that were distributed in nine chromosomes were designed and used in the multiplex PCR reaction system with AmpFLSTR Profiler Plus PCR Kit (Applied Biosystems) (Table S1, http://links.lww.com/MD/D581) (see Table S1, Supplemental Content, which demonstrates the detailed information about the SNPs used in this study). Genomic DNA (1 ng in 10 μl) was added to 15 μl of reaction master mix. PCR operation procedure as follow: 95°C, 10 min→ (95°C, 30s→Tm 60°C↓1°C/cycle, 30s→72°C, 45 s) x10 → (95°C, 30s→Tm 50°C, 30s→72°C, 45 s) x30 →72°C, 10 min→4°C, forever. The productions of multiplex PCRs were sequencing by Hiseq 2500 platform using the SE91 single-end method. Maternal and miscarriage DNA was analyzed in parallel to detect maternal cell contamination (MCC). Levels of MCC were determined by the proportion of non-inherited maternal allele relative to the proportion of the inherited maternal allele. For the purpose of this study, only samples with levels of MCC < 5% were included, so that true chromosomal mosaicism could be clearly differentiated diagnostically from MCC. The fetal allelic SNPs patterns were also used as a secondary validation of aneuploidy detection by NGS. Biallelic (1:1) and triallelic (1:1:1) peaks were indicative of disomy and trisomy, respectively.

2.3. Library construction, sequencing

DNA fragmentation was performed using Covaris M220 according to the manufacturer's protocol, and targeted fragment size was set at 180 bp. DNA fragments were used for sequencing library preparation using NEBNext DNA library prep master mix for Illumina following NEB Next manual (NEB, E6040S). DNA size in the libraries was checked using Agilent 2100 Bioanalyzer. Roche Light Cycler LC480 was employed to evaluate library concentration. Libraries with different indexes were pooled. Sequencing was performed using Illumina PE100 flowcell on Illumina Hiseq2500 platform. For aneuploid analysis, Illumina flowcells (single end with length 41 bp) were used for sequencing by using a low-depth sequencing strategy, about 10 Mb reads each sample.

2.4. General data processing

The mapped reads from NGS were processed through GC correction, normalization, and comparison with the reads in the aneuploid control group in each window (bin) for chromosome aneuploid ratio calculation. Mapped reads were normalized to relative reads number at 10,500/1Mb per bin after GC correction. Aneuploid control consists of data from 187 healthy subjects. The aneuploid analysis was performed using a set of proprietary Perl scripts, visualization was processed with a proprietary R script. In this study, we performed aneuploid Z score analysis. Data from all chromosomes were included in aneuploid Z score analysis. Visual chromosomes change was

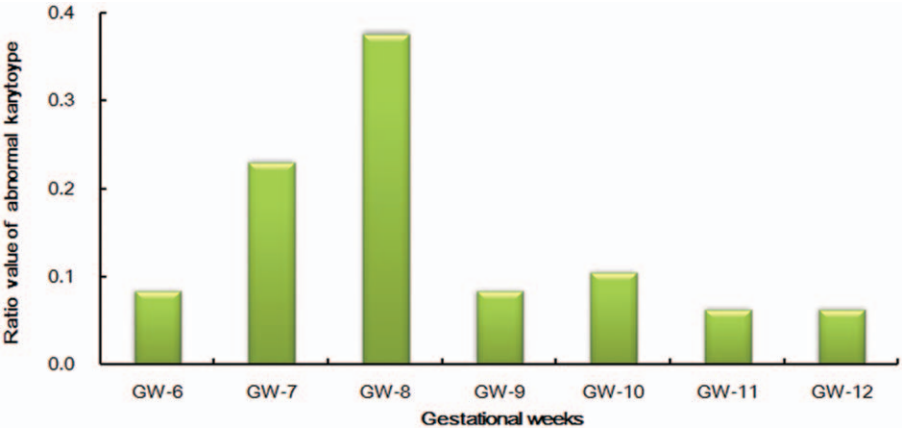

Figure 1. Statistic of gestational weeks of 48 miscarriage cases in this study. The X-axis represents the gestational weeks from 6 weeks to 12 weeks. Y-axis represents the ratio value of abnormal karyotype in each week of all 48 miscarriage cases.

defined as visible intense chromosome increase or decrease in the aneuploid ratio in the 1 Mb per bin aneuploid plots. Scattered dots above or below the baseline were not counted. Read breaks due to repeat sequences (close to centromere or telomere) that appear in every sample were ignored.

3. Results

3.1. Specimen and karyotype analysis

We had counted the gestational age of 48 miscarriage cases which was between 6 and 12 weeks. The results indicated that miscarriage can happen in any period between 6 and 12 weeks of gestational age (Fig. 1). However, the timing of eight weeks gestational age possessed the largest proportion of all samples in this study (37.5%; 18 of 48), which suggested that eight weeks of gestational age may be the most dangerous timing to the most pregnant women. Recent studies have demonstrated that the type of tissue received by a cytogenetics laboratory is critical for the success of cell growth in culture and the subsequent karyotype analysis.[26] Therefore, chorionic villi were selected as culture materials. Moreover, once the samples were received, chorionic villi were separated from the production of conceptions and executed the cell culture immediately in case of any influence on the experimental results. Therefore, these 48 first-trimester spontaneous miscarriages samples had been karyotyped. Our observations demonstrated that the average culture success rate with placental villi was over 58% (58.33%; 28 of 48) (Fig. 2), which was not entirely consistent with those of previous studies.[26] The failures of cell

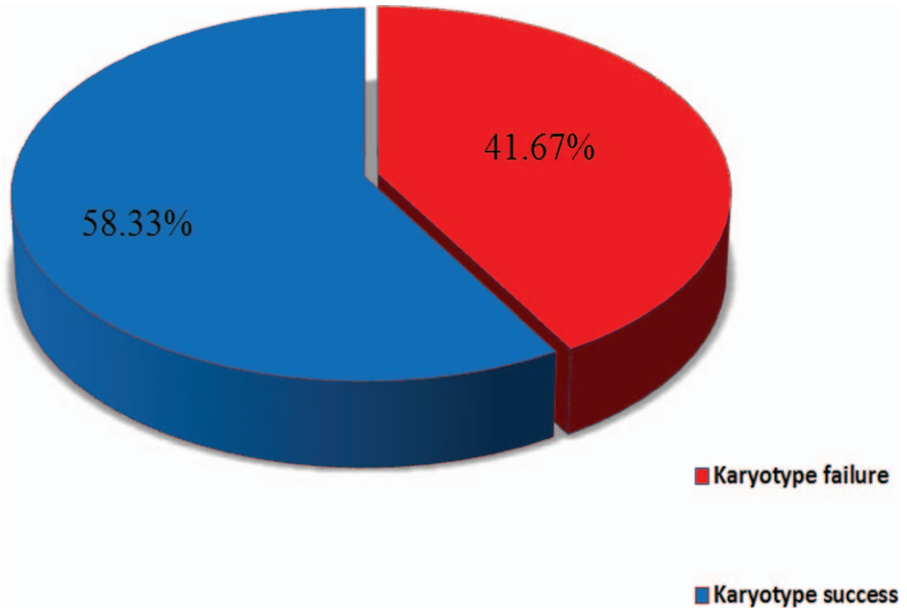

Figure 2. Statistics on the success rate and failure rate of traditional G-band karyotyping. In this study, we have included 48 miscarriage samples. The karyotype results of 28 samples were successful (58.33%, 28 of 48). Meanwhile, The karyotype results of 20 samples were successful (41.67%, 20 of 48).

culture were caused by several factors, including bacterial contamination, none of cell growth, and so on.

3.2. NGS analysis

According to the results of the multiplex PCR analysis against the corresponding maternal genomic DNA samples, none of the 28 miscarriage samples with successful karyotype showed any evidence of significant MCC or polyploidies or haploidy. Figure S1, http://links.lww.com/MD/D580 showed the major allele frequency distribution in a diploid cell without any maternal contamination and the major allele frequency distribution in diploid cell with maternal contamination (see Fig. S1, http://links.lww.com/MD/D580, Supplemental Content, which illustrates the major allele frequency distribution in the diploid cell). In diploid cells without any maternal contamination, the frequency distribution of major allele at heterozygous sites is 0.5. However, in a diploid cell with maternal contamination, the frequency distribution of major allele at heterozygous sites could be ranged between 0.5 and 1.0. Therefore, the 28 samples were coded and sent to the NGS facility for sequencing. Successful results were obtained by NGS in all 28 samples (100%). when the NGS results and karyotype aneuploidy results were compared, it was showed that the NGS identified all abnormalities which were also verified in karyotype examination. NGS had correctly detected 21 of the 21 gross chromosomal abnormalities identified by karyotyping examination, including 9 chromosome 16 trisomies, 3 chromosome 22 trisomies, 2 chromosome 7 trisomies, 2 chromosome 18 trisomies, 1 chromosome 4 trisomies, 1 chromosome 10 trisomies, 1 chromosome 13 trisomies, 1 chromosome 15 trisomies and one sex chromosomal aneuploidies (45, X) (Table 1 and Fig. 3). The Specificity and sensitivity of the NGS combined with multiplex PCR-based chromosome aneuploidy screening was 100% for a normal (7/7) and 100% for abnormal (21/21) results. There were no false-negative diagnoses for aneuploid chromosomes or inaccurate predictions of gender. Comparative graph examples of NGS and karyotype results are shown in Figure 4, in which samples with trisomy were exhibited. All abnormal samples showed balanced, structural abnormalities, that is, gain or loss of entire chromosomes. All of the NGS results were consistent with karyotyping. Interestingly, we had noticed that 16 trisomies had the largest proportion of all 28 successful karyotype cases (32%; 9 of 28), which suggested that 16 trisomies may be an important reason for the first-trimester spontaneous miscarriages.

Table 1

Statistic of 28 miscarriage cases tested by NGS and karyotype.

Research sample	Age of pregnancies	Gestational age	Karyotype	NGS diagnosis
Rs1	26	7	47, XX, +16	47, XX, +16
Rs2	39	7	47, XY, +16	47, XY, +16
Rs3	31	10	47, XX, +16	47, XX, +16
Rs4	23	12	45, X	45, X
Rs5	35	7	47, XY, +16	47, XY, +16
Rs6	26	8	47, XY, +16	47, XY, +16
Rs7	29	9	47, XY, +16	47, XY, +16
Rs8	25	8	47, XY, +16	47, XY, +16
Rs9	30	6	46, XX	46, XX
Rs10	25	9	46, XX	46, XX
Rs11	32	8	47, XY, +22	47, XY, +22
Rs12	27	7	47, XX, +10	47, XX, +10
Rs13	28	9	46, XX	46, XX
Rs14	39	10	47, XY, +18	47, XY, +18
Rs15	36	6	47, XX, +22	47, XX, +22
Rs16	39	7	47, XY, +4	47, XY, +4
Rs17	32	10	47, XX, +16	47, XX, +16
Rs18	33	8	47, XY, +22	47, XY, +22
Rs19	41	8	47, XX, +7	47, XX, +7
Rs20	29	8	46, XX	46, XX
Rs21	28	8	46, XY	46, XY
Rs22	31	7	46, XY	46, XY
Rs23	34	9	47, XX, +7	47, XX, +7
Rs24	27	8	46, XY	46, XY
Rs25	33	11	47, XX, +18	47, XX, +18
Rs26	26	8	47, XY, +13	47, XY, +13
Rs27	36	7	47, XY, +15	47, XY, +15
Rs28	35	7	47, XY, +16	47, XY, +16

4. Discussion

In this study, we evaluated the performance of NGS combined with multiplex PCR based method against traditional cytogenetic karyotyping for the detection of trisomies in spontaneous miscarriage samples. For aneuploid, NGS had a detection rate of 100% (21 of 21), which was similar to array-based methods (86%), which had been used to analysis 314 samples from 9 collective studies.[26,27] Cytogenetic studies had shown that most of these abnormalities were numerical chromosome abnormalities (86%), and a minority of the cases was caused by structural chromosome abnormalities (6%) and chromosome mosaicism (8%).[28] At present, more chromosome abnormalities were found by using fluorescence in situ hybridization (FISH), multiplex ligation-dependent probe amplification (MLPA), and so on. The advantage of using these techniques was that no cell culture was needed, as 20% of the cell cultures failed.[29] Quantitative fluorescent polymerase chain reaction (QF-PCR) was a chromosome region-specific technique but performed as good as conventional karyotyping, and its result depended on the chromosome markers or probes used. When using whole genome techniques, chromosomal-CGH detected an equal amount of chromosome abnormalities (52%) and missed a comparable amount of chromosome abnormalities (6%) compared to karyotyping (60%) and (2%). Array-CGH detected an equal number of chromosome abnormalities (31%) compared to karyotyping (30%) but missed less chromosome abnormalities (2%) compared to karyotyping (10%).[30] This can be explained by the fact that karyotyping had a higher failure rate (18%) compared to array-CGH (5%). Moreover, the costs of the methods mentioned above were higher compared with the gold standard. More importantly, certain abnormalities like polyploidy remain undetectable by chromosomal-CGH, array-CGH and MLPA. QF-PCR can detect polyploidy, but the detection rate of chromosome abnormalities is strongly correlated with the markers used. Molecular techniques like array-CGH, FISH and MLPA may have certain advantages apart from routine cytogenetic analysis of miscarriage samples for investigation of chromosomal abnormalities. FISH and MLPA can detect only preselected submicroscopic abnormalities while array-CGH can detect chromosome abnormalities in the whole genome. In case of culture failure or maternal contamination, molecular techniques may contribute to detect additional chromosome abnormalities in these miscarriages samples in addition to standard karyotyping.[30] Up to now, with the rapid development

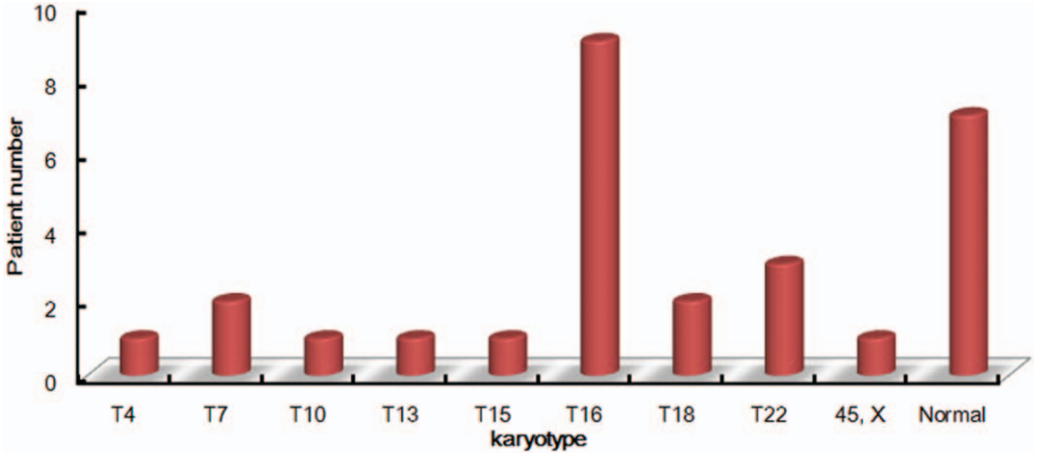

Figure 3. Karyotype information for 28 samples included in the study. T means trisomy, for example, T16 means trisomy of chromosome 16. The X-axis represents the karyotype information of all 28 miscarriage samples. Y-axis means the patient number with different karyotype.

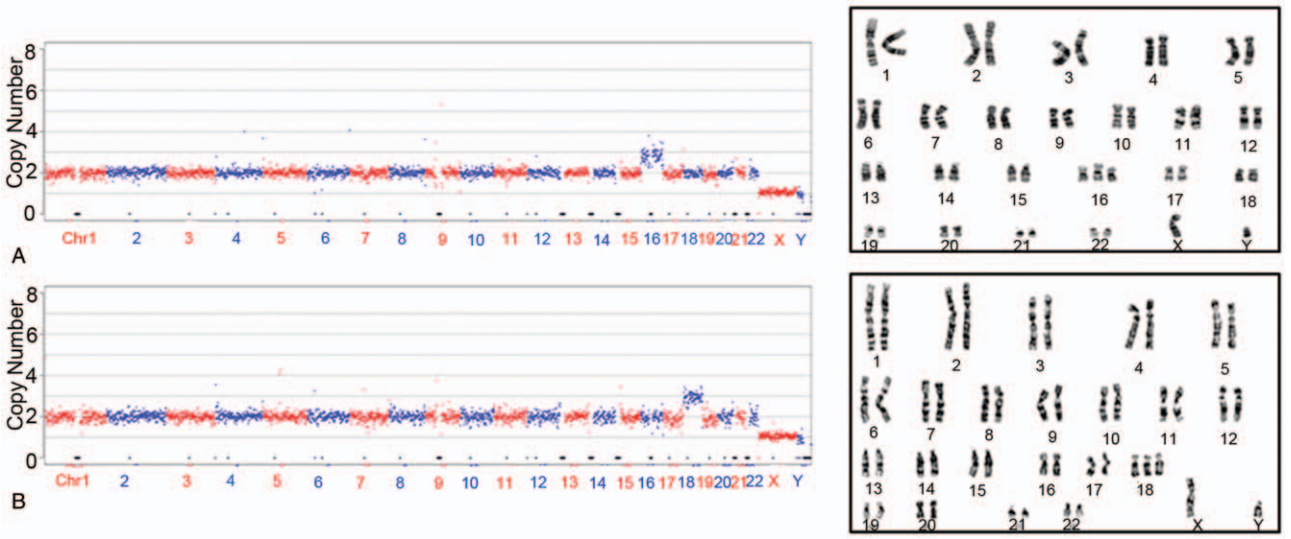

Figure 4. Comparative graph examples of NGS and karyotype results. (A) Trisomy of chromosome 16 was identified by NGS and karyotype. (B) Trisomy of chromosome 18 was verified by NGS and karyotype.

of the next-generation sequencing technologies, NGS offered considerable advantages over arrays and other molecular technologies in terms of a simpler protocol, a faster diagnostic turnaround time (12–16 hours compared to 20–24 hours) and scalability for efficient analysis of multiple samples in the same sequencing batch. In addition, the cost of sequencing is expected to fall over time. NGS techniques will eventually become much more cost-effective than arrays and other methods. More importantly, multiplex PCR detecting SNPs combined with NGS can provide more accurate results. For example, the method mentioned above can detect polyploidy.

Recent studies have demonstrated that the type of tissue received by a cytogenetics laboratory is critical for the success of cell growth in culture and the subsequent karyotype analysis.[31] Previous studies demonstrated that the average culture success rate varies by tissue type with placental villi being the highest (>80%) and fetal parts being the lowest (<40%). Placental decidua almost always represents maternal tissue and is thus not an appropriate specimen type for study. However, the result in this study was not completely consistent with the previous studies. For example, the success rate of karyotype was 58.3%, which is significantly lower than in previous studies.[25] We speculated that the failure of karyotype may be caused by the following two reasons. First, inaccuracy chorionic villus sampling may be the main reason. Although we had executed the most stringent standards for specimen collection, chorionic villi tissue was sampled from different regions of the placenta for karyotyping, which may give rise to the wrong sampling and placenta tissue owned the relatively low rate for cell culture. Second, repeatedly manual operations may introduce bacterial contamination, which can influence the final success rate of karyotyping.

Based on the high diagnostic performance of NGS, our results suggest that NGS combined with multiplex PCR is a viable alternative to karyotyping for detecting chromosomal abnormalities associated with miscarriage. In addition, NGS offers a number of advantages over karyotyping. First, the turnaround time for diagnosis is considerably shorter since chronic villi can be directly analyzed. Whereas, karyotyping requires additional steps such as the preparation of a single cell suspension, extended cell culture, metaphase conversion, and chromosome staining. Second, direct analysis circumvents potential cell culture issues such as "artificial mosaicism" which can occasionally arise from either mitotic error or differential growth rates of the 2 or more cell lines, disguising the true level of mosaicism.[32] Third, since 10 ng of genomic DNA is the minimum template required for NGS to produce an accurate result, NGS technologies may be a more reliable technology, particularly in clinical situations where only small amounts of tissue are retrieved for analysis, which may be insufficient for successful cell culture and karyotyping.[33] Despite these advantages, karyotyping still remains the gold standard for the detection of common aneuploidies, polyploidies, and mosaicism in miscarriage samples with a relatively low cost. In regard to arrays, NGS has been demonstrated to perform similarly to SNP arrays for detection of chromosomal abnormalities in patient samples, including whole and partial aneuploidies.[34] Further, based on this NGS study and previous studies using different types of arrays, both techniques have demonstrated the ability to detect aneuploidies in miscarriage tissues.[27] One advantage of the current method is that in contrast to SNP arrays, polyploidies, which are also another significant cause of miscarriage, can be detected (exception 69, XXY and 69 XYY). At the laboratory level, NGS offers considerable advantages over arrays in terms of a simpler protocol, faster diagnostic turnaround time and scalability for efficient analysis of multiple samples in the same sequencing batch. In addition, as the cost of sequencing is expected to fall over time, NGS techniques will eventually become much more cost-effective than arrays. In order to provide optimized, cost-effective care for couples with miscarriage, it is important to determine whether the pregnancy was miscarried as the result of a chromosomal abnormality or possibly another factor. A positive result has a direct impact on genetic counseling for the couple. Meanwhile, a negative result can direct the in-depth evidence-based workup for endocrinologic, hematologic, anatomic, or immunologic imbalances in the mother.

NGS offers an alternative technology to karyotyping and arrays for accurately identifying the known chromosomal causes associated with spontaneous miscarriages. In addition, NGS has a relatively high resolution across the genome and the ability to precisely quantitate copy number. Therefore, NGS based technology could provide more information about the copy number variants (CNVs) with miscarriage of the first trimester.

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Author contributions

Chen Min, Liu Qi Yun, Hu Shun Qin, Li Li Rui, and Li Jia participated in the whole experimental processes. Ma Run Mei has provided technical assistance. Xu Jing and Ma Run Mei have written the manuscript.

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