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Original Article

Improved assay performance of single nucleotide polymorphism array over conventional karyotyping in analyzing products of conception

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Abstract

Background: Conventional karyotyping has been a routine method to identify chromosome abnormalities in products of conception. However, this process is being transformed by single nucleotide polymorphism (SNP) array, which has advantages over karyotyping, including higher resolution and dispensing with cell culture. Therefore, the purpose of this study was to evaluate the advantage of high-resolution SNP array in identifying genetic aberrations in products of conception.
Methods: We consecutively collected 155 products of conception specimens, including 139 from first-trimester miscarriage and 16 from second-trimester miscarriage. SNP array was performed on these samples in parallel with G-banded karyotyping.
Results: The test success rate was 98.1% (152/155) using SNP array, which was higher than that using karyotyping (133/155, 85.8%). It yielded a 63.8% (97/152) abnormality rate, and the frequency of various chromosome abnormalities was in agreement with other previous studies. The results between array and karyotyping demonstrated a 94.0% (125/133) concordance. SNP array obtained additional aberrations in 3.8% (5/133) of those cases unidentified by karyotyping, which included three cases with whole-genome uniparental disomy, one with pathogenic copy number variation, and one with del(4)(q35.1q35.2) and dup(12)(q24.31q24.33). However, chromosome translocations presented in two cases and tetraploidy presented in one case were detected by karyotyping instead of array. Additionally, two out of three cases with mosaic trisomy were revealed by array but recognized as pure trisomy by karyotyping.
Conclusion: This study demonstrated that SNP array had certain advantages over G-banded karyotyping, including a higher success rate, additional detection of copy number variations and uniparental disomy, and improved sensitivity to mosaicism. Therefore, it would be an alternative method to karyotyping in clinical genetic practice.

Keywords: copy number variation; karyotyping; products of conception; single nucleotide polymorphism array; uniparental disomy

1. Introduction

Miscarriage is the spontaneous loss of pregnancy before 24 completed weeks of gestation.[1] Approximately 10–15% of all clinically recognized pregnancies end in miscarriage, mostly during the first trimester (up to 12 weeks' gestation).[2] Although many risk factors relate to this occurrence, genetic factors play the most important role. In addition, cytogenetic analysis of products of conception (POC) from miscarriage

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has indicated that severe genomic imbalance caused by embryonic chromosome abnormalities accounts for approximately 50% of first-trimester miscarriage, of which there were 86% numerical abnormalities, 6% structural abnormalities, and 8% other chromosome abnormalities such as mosaicism or double and triple trisomies.[3] In the past several decades, chromosomal karyotyping has been used as the standard to detect microscopic chromosome aberrations in POC. It is crucial for chromosomal karyotyping to collect sterile specimens with sufficient viable cells of the villi, which often depends on the duration of embryo or fetus death, and the manner of acquiring POC samples. In addition, because of its technique limitations, such as high rate (5–42%) of tissue-culture failure,[4] poor chromosome preparations, possible maternal cell contamination, low resolution, and high turn-around time, the genetic causes of miscarriage have not been fully elucidated in clinical practice. Recently, however, chromosomal microarray analysis (CMA), including array-based comparative genomic hybridization (array-CGH) and single nucleotide polymorphism (SNP) array, is increasingly being applied to the analysis of POC. Due to the advantages of reduced requirements regarding sample quality and improved resolution, DNA-based CMA may overcome many of the limitations existing in karyotyping. Previous studies using array-CGH for investigation of POC indicated that array-CGH could detect an additional 5% of copy number variations (CNVs) that remained undetectable for karyotyping.[2,5–10]

Furthermore, Lathi et al[11] successfully applied another CMA platform of SNP array to the analysis of 30 POC specimens, and reached 87% accordance with G-banded karyotyping. Recently, Levy et al[12] found that clinically significant CNVs and whole-genome uniparental disomy (UPD) in 1.6% and 0.4% of POC specimens, respectively, by also using SNP array. However, in contrast to karyotyping, there are relatively few applications of SNP array in the clinical investigation of POC. As limited data are available on the clinical utility of CMA as a routine method to evaluate first- and second-trimester miscarriage,[13] more studies are required for assessing its value of clinical routine application at this time. Therefore, to evaluate the advantage of high-resolution SNP array in identifying genetic aberrations in POC, this study compared it with conventional G-banded karyotyping by analyzing 155 POC samples derived from first- and second-trimester (12–24 weeks' gestation) miscarriage.

2. Methods

2.1. Cases

A total of 155 cases diagnosed as missed abortion underwent the dilation and curettage procedure from July 2013 to April 2014. All 155 POC specimens, including 139 from first-trimester miscarriage (mean gestational age was 8.9 weeks, ranging from 6 weeks to 12 weeks) and 16 from second-trimester miscarriage (mean gestational age was 15.9 weeks, ranging from 13 weeks to 19 weeks) were collected consecutively. Among 139 cases from first-trimester miscarriage, 95 were diagnosed with a missed miscarriage and 44 with a blighted ovum. All women had spontaneous conception with singleton pregnancy. The mean age of women was 32.2 years, with a range 22–45 years. Of the 155 cases, 116 cases were without prior miscarriages or with one prior miscarriage (also called sporadic miscarriage), 32 cases had equal to or more than two prior miscarriages (also called recurrent miscarriage), and seven had an unknown history of miscarriage. Prior to the initial study, the Medical Ethics Committee of our hospital checked and approved the research program. All women gave consent for the execution of the cytogenetic and molecular cytogenetic investigation on the POC and signed written informed consent forms saved in medical records after being reviewed by the Medical Ethics Committee.

2.2. Specimen preparation

We examined POC specimens grossly in part for the purpose of excluding specimens that consisted only of placental decidua and, thereafter, acquired a small portion of chorionic villi. Then it was washed with 0.9% normal saline at least three times to clean out visible blood clots, and separated by forceps from maternal decidua, blood vessels, blood clots, membrane, and other materials under a dissecting microscope. Thereafter, another laboratory technologist performed a similar procedure on the preliminary selected samples, to ensure that most fetal chorionic villi were isolated from maternal tissues.

Although SNP array is supposed to reveal potential maternal cell contamination in POC by acquiring parental specimens that are analyzed simultaneously, it would be unaffordable to patients and difficult to perform in a clinical environment. Therefore, we did not adopt this means in our study. However, it was expected that the potential maternal cell contamination in selected villus samples would be minimal in our sample processing technique, as referenced above.

Each selected chorionic villus was cut into pieces and then divided into three parts: one-third was used for cell culture and subsequent G-banded karyotyping or metaphase fluorescence in situ hybridization (FISH), one-third for DNA extraction and SNP array analysis, and the remaining one-third was disaggregated using 60% acetic acid and stored at −20°C after being fixed by methanol/acetic acid (ratio 3:1) for interphase FISH.

2.3. Conventional karyotyping analysis

Cell culture and routine G-banded karyotyping were performed according to standard protocols. The International System for Human Cytogenetic Nomenclature 2009 was utilized to help define chromosome abnormalities. In addition, karyotyping results were blinded to the experimenters before SNP array analysis was performed.

2.4. SNP array analysis

Genomic DNA was isolated from uncultured chorionic villus cells with QIAamp DNA mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. DNA samples

(250 ng) were hybridized to Affymetrix CytoScan 750K arrays following the manufacturer's protocol. The Affymetrix CytoScan 750K array contains >750,000 markers for copy number analysis, of which 550,000 are unique nonpolymorphic oligonucleotide probes and 200,000 SNP probes that can be used for genotyping. Average marker spacing is one probe every 4.1 kb, with a mean spacing of one probe every 6.1 kb on nongene backbone and one probe every 1.7 kb in intragenic regions. Aberrations were filtered by the Affymetrix ChAS 2.0 software up to a minimal size of 200 kb and at least 50-probe calls for deletions and duplications. ChAS was designed to reliably detect only mosaicism between approximately 30% and 70% for CNVs $\geq$ 5 Mb in size. In addition, this software for 750K arrays was set for displaying loss of heterozygosity of $\geq$ 3 Mb in size, and a cutoff of a single loss of heterozygosity of > 10 Mb on a single chromosome was used for indicating UPD. Results were analyzed manually for CNVs and loss of heterozygosity. All data had to meet the Quality Control (QC) metrics including SNPQC $\geq$ 15, Median of the Absolute values of all Pairwise Differences (MAPD) $\leq$ 0.25, and waviness standard deviation (SD) $\leq$ 0.12. A cutoff of 10 Mb in size was used to distinguish CNV ($\leq$ 10 Mb) from cytogenetically chromosomal deletion or duplication (> 10 Mb).[14]

We compared all observed CNVs with those catalogued in the Database of Genomic Variants (DGV) and the Database of Chromosomal Imbalance and Phenotype in Humans using Ensemble Resources (DECIPHER), and then classified CNVs as common CNVs and unique CNVs. Common CNVs were defined as those that overlapped completely with CNVs reported in at least one study catalogued in the DGV with > 100 cases studied and a frequency of $\geq$ 1%, or at least two studies catalogued in the DGV. Unique CNVs were defined as those that showed no or incomplete overlap with CNVs in the DGV, or that are presented in the DGV, but with < 100 cases studied and/or a frequency of < 1%.[5,15] The building of the human genome assembly was based on GRCH37/hg19. Unique CNVs were assessed by searching for similar cases in DECIPHER and PubMed, and also by searching for gene information in Entrez Gene and OMIM. We also classified unique CNVs according to categories of clinical significance recommended by the American College of Medical Genetics. It categorizes CNVs into pathogenic variations, benign variations, and variations of uncertain clinical significance (VOUS), and VOUS can be further subdivided into likely pathogenic, likely benign and VOUS.[16]

Besides, the results of mosaicism or pathogenic CNVs were verified by interphase or metaphase FISH.

2.5. Statistical analysis

SPSS version 13 for Windows (SPSS Inc., Chicago, IL, USA) was utilized to perform statistical analysis of the Chi-square test. A p value < 0.05 was considered statistically significant.

3. Results

G-banded karyotyping and SNP array were performed on 155 POC samples concurrently. Finally, karyotyping was successfully performed on 133 cases but failed in 22 cases due to failed cell culturing, while SNP array was successfully analyzed in 152 cases and failed in three cases because of substandard QC metrics, which probably resulted from poor DNA quality. Overall, SNP array yielded a 63.8% (97/152) abnormality rate. The abnormality rate (84/116, 72.4%) in sporadic miscarriage cases was higher than that rate (13/32, 40.6%) in recurrent miscarriage cases ($p = 0.001$). However, SNP array results either from first-trimester (90/136, 66.2%) and second-trimester (7/16, 43.8%) miscarriage ($p = 0.077$), or from missed miscarriage (62/92, 67.4%) and blighted ovum (28/44, 63.6%) ($p = 0.665$) showed no difference. Fig. 1 demonstrated the frequency of various aberrations detected by SNP array. Of these abnormalities ($n = 97$), the most common were aneuploidies, primarily including trisomy 16 (23.7%, 23/97), monosomy X (11.3%, 11/97), trisomy 22 (10.3%, 10/97), triploidy (6.2%, 6/97), trisomy 13 (5.2%, 5/97), trisomy 21 (4.1%, 4/97), trisomy18 (4.1%, 4/97), and trisomy 4 (4.1%, 4/97).

The overall test success rate was 98.1% (152/155) in SNP array and 85.8% (133/155) in karyotyping. A total of 133 cases were successfully analyzed by SNP array and karyotyping, and the results demonstrated 94.0% (125/133) concordance. Discrepancies of results between SNP array and karyotyping occurred in eight cases. SNP array revealed all imbalanced chromosomal abnormalities and detected additional aberrations in five cases (5/133, 3.8%) unidentified by karyotyping, which included one case with pathogenic CNV (1/133, 0.8%), three cases with whole-genome UPD (3/133, 2.3%), and one case (1/133, 0.8%) with a 4.3-Mb del(4)(q35.1q35.2) and a 11.7-Mb dup(12)(q24.31q24.33). However, balanced reciprocal translocations presented in two cases, for which karyotypes were 46,XX,t(11;15)(p15.2;q11.2) and 46,XY,t(12;13)(p13;q22), and tetraploidy, which presented in one case, was detected by karyotyping instead of array. Additionally, two out of three cases with mosaic aneuploidy were revealed by array but recognized as pure trisomy by karyotyping (Table 1); another case was detected as 48,XXY, +9/47,XXY mosaicism by both methods. Among the 22 cases with failure of cell culture due to bacterial pollution or no growth, SNP array succeeded in 86.4% (19/22) cases and yielded a 50.0% (11/22) abnormality rate.

In the SNP array study series, among 56 cases without chromosome abnormalities ($n = 93$) and UPD ($n = 3$), 49 were found to be of various CNVs, with a total number of 95. Thirty-eight out of the 95 CNVs (38/95, 40.0%) were classified as unique CNVs and 57 (57/95, 60.0%) as common CNVs. Of the 38 unique CNVs (Tables 2 and S1), five were recognized as likely benign CNVs due to the absence of genes, one was considered as likely pathogenic CNV, and the other 32 were classified as VOUS. The likely pathogenic CNV was located at 22q11.21 (18,919,477–21,800,471), completely overlapping the known 22q11.2 microdeletion syndrome region. We confirmed this CNV as a *de novo* deletion after performing FISH on both POC and peripheral blood from parents.

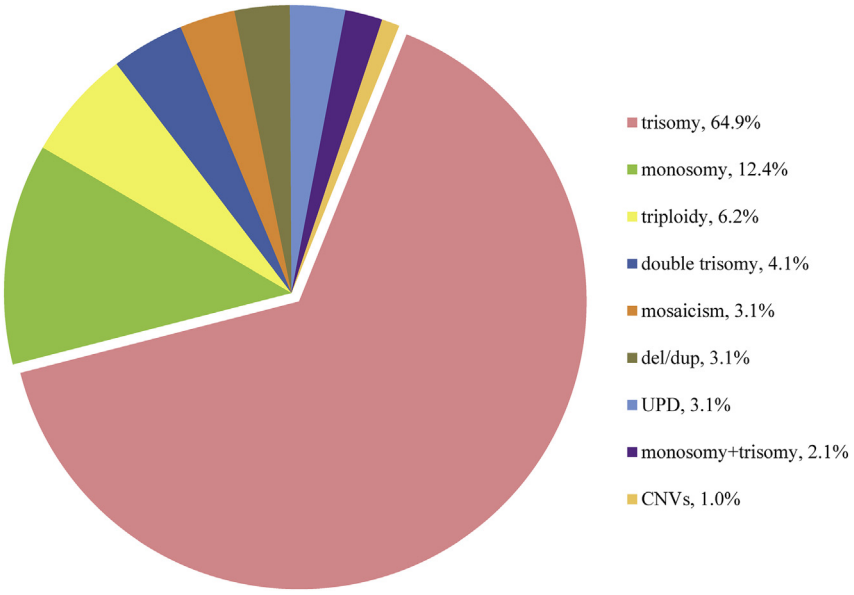

Fig. 1. Frequency of various abnormalities detected by SNP array. Trisomy was the major abnormality, accounting for 64.9% (63/97) of all abnormalities. Monosomy accounted for 12.4% (12/97), including monosomy X ($n = 11$) and monosomy 21 ($n = 1$). Other chromosome abnormalities accounted for 23% (18/97), including triploidy ($n = 6$), double trisomy ($n = 4$), mosaicism ($n = 3$), deletion/duplication ($n = 3$), monosomy plus trisomy ($n = 2$). Whole-genome UPD ($n = 3$) and likely pathogenic CNVs ($n = 1$) accounted for 3.1% and 1.0%, respectively. CNV = copy number variation; SNP = single nucleotide polymorphism; UPD = uniparental disomy.

Table 1
Comparison of SNP array and karyotyping results in 155 POC samples.

		G-banded karyotyping			
		Normal	Chromosomal abnormality	Failure	Total
SNP array	Normal	44	3[a]	8	55
	Chromosomal abnormality	1[b]	81[c]	11	93
	Pathogenic CNV	1	0	0	1
	UPD	3	0	0	3
	Failure	0	0	3	3
	Total	49	84	22	155

CNV = copy number variation; POC = products of conception; SNP = single nucleotide polymorphism; UPD = uniparental disomy.
[a] Two cases with balanced reciprocal translocation and one case with tetraploidy.
[b] Arr 4q35.1q35.2 (186,511,421–190,806,055) × 1, 12q24.31q24.33 (122,091,931–133,777,562) × 3.
[c] One case with arr(2) × 2.35, (X) × 2, and one case with arr(18) × 2.37, (XY) × 1 were not diagnosed as mosaicism, but as trisomy 2 and trisomy 18 separately by karyotyping.

Table 2
Summary of 38 unique CNVs detected in 30 cases with normal karyotypes.

Description	Properties	CNV count
Size (210–2881 kb)	200–400 kb	18
	400–1000 kb	13
	1–2.88 Mb	7
Gene content	≥ 1 coding gene	33
	≥ 1 OMIM gene	16
	No gene	5
Chromosomal location	Autosome	35
	Sex chromosome	3
Type of CNV	Duplication	31
	Deletion	6
	Amplification	1

CNV = copy number variation.

4. Discussion

Compared with conventional G-banded karyotyping, we employed SNP array to identify genetic aberrations in 155 POC specimens. Overall, both methods showed 94.0% (125/133) concordance, but the SNP array achieved a higher detection success rate than karyotyping (98.1% vs. 85.8%) and enabled the detection of submicroscopic chromosomal abnormalities and UPD. Many factors, including cell culture failure, poor chromosome morphology, selective growth of abnormal cell lines or maternal cells, and subjective error of technicians, could influence the success rate or accuracy of conventional karyotyping. In our study, it seemed that the lower success rate of karyotyping mainly came from cell culture failure because of bacterial contamination or decreased

cell viability, which were nearly unavoidable in certain POC specimens. However, DNA-based array using uncultured cells could overcome these limitations, as shown in this study that SNP array succeeded in 86.4% of cases that failed in cell culture. SNP array obtained 3.8% additional aberrations over karyotyping, including whole-genome UPD, pathogenic CNV, and small chromosomal deletion/duplication in 2.3%, 0.8%, and 0.8% cases, respectively. Frequencies of additional aberrations were similar to those reported in previous studies that were based on array-CGH or SNP array methods.[2,12]

In general, there was an agreement between array and karyotyping results on frequencies of various chromosome abnormalities, of which aneuploidy was the primary abnormality detected in POC. These were not different from those of previous studies. However, discrepancies between SNP array and karyotyping results occurred in cases with CNVs, UPD, mosaicism, tetraploidy, deletion/duplication, and balanced reciprocal translocation.

Normal embryonic development is controlled by genes. CNV can convey phenotypes through influencing gene expression levels, which results from whole gene copy number change or disruption of part of a gene.[17] Several studies have indicated that CNVs existed in 1–13% of miscarriage cases.[6–10,18] Recently, two studies showed that potentially pathogenic CNVs were present in 6–15% of POC samples by analyzing the content and function of certain genes within CNVs.[5,15] Another study using SNP array to investigate a large-scale cohort of POC samples revealed potentially pathogenic CNVs in 1.6% of cases.[12] However, at present, because data about clinical significance of most CNVs were determined through genotype–phenotype correlation of individuals with clinical features, it was difficult to define whether or not CNVs were pathogenic in most POC specimens without any recognizable phenotypes. Some cases of VOUS were taken as pathogenic because they could not be proved to be benign in many previous studies.[19] In this study, 38 unique CNVs were identified, but 32 were classified as VOUS. It was based on such opinions that no CNV databases or literature reported these CNVs as pathogenic CNVs related to miscarriages or human developmental disorders. However, some of these CNVs carried genes related to embryonic development in biological function and others carried genes of unknown function.[16] Only one *de novo* CNV loss of 2.88 Mb at 22q11.21 was considered to be a likely pathogenic CNV. It completely overlapped the known pathogenic 22q11.2 microdeletion syndrome, which encompasses the phenotypes of DiGeorge syndrome and velocardiofacial syndrome, including but not limited to developmental delay and major congenital heart disease. At present, however, because no case with 22q11.2 microdeletion syndrome had been reported to be associated with human miscarriage, its relationship with miscarriage was still not clear. Sufficient evidence is lacking to confirm that miscarriage was caused by the change of various genes within this region in a correlative way. Therefore, for purposes of identifying the clinical significance of CNVs found in POC, further prospective studies are required to reveal and accumulate information on CNVs.

UPD is defined as the inheritance of both homologs of a chromosome pair from a single parent with no representative copy inherited from the other parent.[20] SNP array, as a genome-wide scanning tool based on SNP genotyping ability, would detect the majority of cases of UPD.[20] Some types of UPD are associated with imprinting disorders, such as Prader–Willi syndrome, Angelman syndrome, and Beckwith–Wiedemann syndrome, which often cause developmental delay, intellectual disability, and malformations. However, UPD also causes abnormal embryonic/fetal development or even miscarriage, although the frequency of UPD-associated miscarriage may be low, which was estimated to occur in < 3% of miscarriage cases.[21,22] Several studies found that UPDs, for example, with maternal uniparentalheterodisomy of chromosome 9,[21] paternal UPD of chromosome 14,[22] maternal uniparentalisodisomy and uniparentalheterodisomy of chromosome 16,[23] and paternal uniparentalisodisomy of chromosome 21,[21] could cause miscarriage. Moreover, it was well known that whole-genome UPD was not compatible with life, and paternal origin would lead to complete hydatidiform mole, resulting in embryonic death.[24] Although most cases of hydatidiform mole could be identified by ultrasonography, very few cases would cause confusion under ultrasound during early pregnancy. As shown in our study, ultrasonography made questionable diagnoses of embryo damage and villi edema in three cases. Hence, their POC samples were sent to our laboratory for SNP array analysis and were finally identified as whole-genome UPD. Being limited by insufficient specimens, we were unable to investigate the parental source of UPD further. However, other parts of these POC were simultaneously sent for pathological examination, and they were confirmed to be complete hydatidiform moles, demonstrating the paternal origin of the three UPD indirectly.

Three cases with mosaicism were detected by SNP array, while only one could be detected by karyotyping with a lower proportion of mosaic. In addition, FISH also confirmed the mosaicism. Two missed cases of mosaicism underlined two important limitations of karyotyping, including the disadvantage of cell culture and subjective error of karyotyping analysis.[6] Subjective errors may result from fewer metaphase cells or poor metaphase spreads. In such a situation, some aberrations are often selectively ignored or could not be found by the reviewing technician. As shown in this study, one case with 4.3 Mb deletion at 4q35.1q35.2 and 11.68 Mb duplication at 12q24.31q24.33 was missed by routine G-banded karyotyping due to poor chromosome morphology or lower resolution, although resolution of G-banding could reach 5–10 Mb in theory. Moreover, selective growth of maternal cells or abnormal cells occurring during cell culture may result in false mosaicism, true mosaicism with incorrect proportion, or even inaccurate karyotype.[6,25] Therefore, these also demonstrated that SNP array was more objective and sensitive than karyotyping in the detection of mosaicism. Notably, it was reported that only 23% of mosaicism detected in POC was confirmed in fetuses, and most mosaicism was shown to be

confined placental mosaicism.[26] Although confined placental mosaicism is difficult to distinguish from true mosaicism in clinical practice, it should be taken into consideration for further investigation and interpretation of results.

Based on genotyping ability, SNP array not only identified UPD, but also triploidy and some types of tetraploidy. In our study, one case with tetraploidy was detected by karyotyping, but not by array. According to different parental origins, tetraploidy can be divided into two different types: 2:2 tetraploidy (more common) and 3:1 tetraploidy (less common). There is a limitation that SNP array is able to detect 3:1 tetraploidy (3 sets of chromosomes from 1 parent and 1 from the other) but not 2:2 tetraploidy.[11] Hence, it might be speculated that tetraploidy present in this case was consistent with the type of 2:2 tetraploidy.

Overall, this study demonstrated that SNP array had certain advantages over G-banded karyotyping in the detection of CNVs, UPD, and mosaicism, but had disadvantages in detection of balanced structural abnormalities and most tetraploidy. Therefore, since SNP array achieved a higher success rate in the experiments and yielded more information about genetic aberrations in POC, it can be considered an alternative method to karyotyping.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jcma.2015.03.010.

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