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Application of Copy Number Variation Sequencing in Genetic Analysis of Miscarriages in Early and Middle Pregnancy

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Keywords
CNV-seq · Spontaneous abortions · Genetic diagnosis · Copy number variation · Aneuploidy

Abstract
High-throughput sequencing based on copy number variation (CNV-seq) is commonly used to detect chromosomal abnormalities. This study identifies chromosomal abnormalities in aborted embryos/fetuses in early and middle pregnancy and explores the application value of CNV-seq in determining the causes of pregnancy termination. High-throughput sequencing was used to detect chromosome copy number variations (CNVs) in 116 aborted embryos in early and middle pregnancy. The detection data were compared with the Database of Genomic Variants (DGV), the Database of Chromosomal Imbalance and Phenotype in Humans using Ensemble Resources (DECIPHER), and the Online Mendelian Inheritance in Man (OMIM) database to determine the CNV type and the clinical significance. High-throughput sequencing results were successfully obtained in 109 out of 116 specimens, with a detection success rate of 93.97%. In brief, there were 64 cases with abnormal chromosome numbers and 23 cases with CNVs, in which 10 were pathogenic mutations and 13 were variants of uncertain significance. An abnormal chromosome number is the most important reason for embryo termination in early and middle pregnancy, followed by pathogenic chromosome CNVs. CNV-seq can quickly and accurately detect chromosome abnormalities and identify microdeletion and microduplication CNVs that cannot be detected by conventional chromosome analysis, which is convenient and efficient for genetic etiology diagnosis in miscarriage. © 2021 S. Karger AG, Basel

Introduction

The incidence of spontaneous abortions is high in clinical pregnancies [Hailu and Kebede, 1994]. Studies have reported genetic factors to play a leading role in early abortion, with approximately 50% of cases caused by chromosomal abnormalities [van den Berg et al., 2012]. Presently, autosomal trisomy is the most common cause of early pregnancy abortion (65%), followed by triploidy (13%) and 45,X (10%) [Muñoz et al., 2010; Soler et al., 2017], whereas copy number variations (CNVs) cause approxi-

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Table 1. CNV-seq test results of aborted tissues

Cases	Normal	Single chromosome aneuploidy	Double chromosome trisomy	Chimeric chromosome aneuploidy	Polyploidy	70,XXY,+21	Pathogenic CNVs	VOUS	Total
Number, *n*	22	50	4	7	2	1	10	13	109
Proportion, %	20.18	45.87	3.67	6.42	1.83	0.92	9.17	11.93	100.00

mately 2.2% of miscarriages [Wang et al., 2017]. In the remaining 50% of spontaneous or recurrent miscarriage cases, the cause is closely related to an environmental or genetic factor or a history of endocrine or immune diseases, including anatomical abnormalities and acquired thrombosis [Brown, 2008]. Regardless of the cause, etiology analysis of the abortion has a significant impact on the woman's next pregnancy [Borrell and Stergiotou, 2013].

Although karyotype analysis is still the gold standard for identifying chromosomal abnormalities, this technique cannot effectively analyze CNVs <10 Mb [Pasquier et al., 2016]. By contrast, techniques such as fluorescence in situ hybridization, quantitative fluorescent polymerase chain reaction, and multiplex ligation-dependent probe amplification are commonly used to verify clinical findings [Riegel, 2014]. Compared with karyotype analysis, comparative genomic hybridization and single nucleotide polymorphism (SNP) arrays have a greater resolution (approximately 0.1 Mb), and thus, they are used as the main diagnostic methods [Bug et al., 2014] or combined with karyotype analysis to further identify the chromosomal causes of miscarriage [Dhillon et al., 2014]. In recent years, next-generation sequencing (NGS) has been introduced for chromosome analysis [Ong et al., 2013]. This high-throughput sequencing approach is highly accurate and sensitive, as well as economical, so it is commonly used in the identification of CNVs [Liang et al., 2014]. In this study, we applied CNV-seq to villous and fetal tissues of 116 patients with unexplained abortion and explored the value of this technology in the detection of abortion causes.

Materials and Methods

A total of 116 specimens from pregnant women with spontaneous abortions who were admitted to the Liuzhou Maternity and Child Healthcare Hospital from April 2017 to September 2019 were collected. All patients enrolled in the study had spontaneous abortions, and recurrent abortions were not excluded. Pregnant women voluntarily underwent CNV-seq testing in abortion clinics, and all patients provided signed informed consent. The age of the pregnant women ranged from 17 to 44 years, with an average age of 33.1 ± 6.0 years. Duration of gestation ranged from 5 to 26 weeks.

CNV-Seq Detection and Data Analysis

Approximately 5–10 mg of villous tissue was selected under a microscope and minced into pieces. A sample of fetal skin tissue was also selected, minced into pieces, and rinsed with sterile PBS buffer. DNA was extracted from both samples. Using a nucleic acid analyzer (model ASP-2680), the purity of the DNA concentration ranged from 1.6 to 1.9, and the concentration ranged from 20 to 30 ng/μL.

After extraction of genomic DNA from aborted tissues, a sequencing library was constructed using the Xromate Kit (Berry Genomics Co., Ltd., Beijing, China). The sequencing library was labeled with Index and then sequenced using the Illumina Hiseq2000 System (San Diego, CA, USA). In brief, 50 ng of genomic DNA was fragmented to an average size of 300 bp and end-ligated with barcoded sequence adaptors. To generate sequencing libraries, tagged DNA fragments were amplified using primers with partial adaptor sequences. CNV libraries were generated after DNA purification and subsequently sequenced on the HiSeq2000 System to generate approximately 5 million 36-bp single-end reads. To evaluate chromosomal copy number, the raw data were analyzed as previously described. The Xromate Data Analysis System was used for data analysis. Thereafter, millions of independent DNA sequences detected within a given sample were matched to the chromosome on which they were located, and standardization analysis was performed to determine the chromosomal abnormality by calculating the Z value. The test data were compared with data in public databases, including the Human Genome Database (version hg19), the Database of Genomic Variants (DGV), the Database of Chromosomal Imbalance and Phenotype in Humans using Ensemble Resources (DECIPHER), the Online Mendelian Inheritance Database in Man (OMIM), the University of California Santa Cruz Database (UCSC), the Orphanet Database, and PubMed to determine the CNV type and clinical significance. In addition, short tandem repeat analysis (model ABI 3500Dx; Foster City, CA, USA) was used to investigate the possibility of maternal contamination of aborted sequencing samples.

Results

CNV-Seq Test Results

A total of 109 out of 116 samples collected in this study underwent successful CNV-seq, with a success rate of

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Table 2. Summarized CNV-seq results

Case number	CNV-seq (hg19 chr:location)	Size, Mb	Clinical significance	Syndrome
17SC14956	seq[hg19]dup(4)(p16.3) chr4:g.360001_560000dup	0.2	VOUS	–
18SC01802	seq[hg19]dup(X)(p11.1) chrX:g.58160001_58480000dup	0.32	VOUS	–
18SC01892	seq[hg19]dup(X)(q23) chrX:g.112380000_112620000dup	0.24	VOUS	–
	seq[hg19]dup(4)(p12) chr4:g.45140001_45560000dup	0.42	VOUS	–
18SC16827	seq[hg19]dup(6)(q12) chr6:g.64660001_65500000dup	0.84	VOUS	–
18SC41563	seq[hg19]del(6)(q12) chr6:g.66500001_66620000del	0.2	VOUS	–
	seq[hg19]dup(2)(q33.1q33.2) chr2:g.203260001_203460000dup	0.12	VOUS	–
18SC41580	seq[hg19]dup(Y)(q11.221) chrY:g.16180001_16320000dup	0.14	VOUS	–
18SC41599	seq[hg19]del(16)(p13.2p13.13) chr16:g.10460001_10820000del	0.20	VOUS	–
	seq[hg19]dup(6)(p22.3p22.2) chr6:g.25020001_25220000dup	0.36	VOUS	–
19SC01154	seq[hg19]del(8)(p22) chr8:g.13380001_13540000del	0.16	VOUS	–
19SC01191	seq[hg19]del(8)(p22) chr8:g.14000001_14100000del	0.10	VOUS	–
	seq[hg19]dup(X)(q28) chrX:g.154620001_154940000dup	0.32	VOUS	–
	seq[hg19]dup(12)(q11q12) chr12:g.37980001_38300000dup	0.32	VOUS	–
19SC01193	seq[hg19]del(2)(p24.1) chr2:g.23100001_23360000del	0.26	VOUS	–
19SC01196	seq[hg19]del(14)(q21.1) chr14:g.41160001_41680000del	0.28	VOUS	–
	seq[hg19]dup(2)(p24.2) chr2:g.17940001_18220000dup	0.52	VOUS	–
19SC15095	seq[hg19]del(6)(q16.3) chr6:g.102700001_104100000del	0.12	VOUS	–
19SC35298	seq[hg19]dup(9)(p23p22.3) chr9:g.14140001_14360000dup	0.22	VOUS	–
17SC14686	seq[hg19]dup(8)(q11.1q24.3)(mos) chr8:g.47680001_140580000dup	92.9	Pathogenic	Chromosome 8q22.1 duplication syndrome [OMIM #151200]
17SC14953	Xp22.31(6700001_8080000)	1.38	Pathogenic	Stunting [Patient:287718] [Patient:300318]
17SC14958	seq[hg19]dup(22)(q11.1q11.23) chr22:g.16840001_25720001dup	8.88	Pathogenic	Like trisomy 22 symptoms [Patient:300318]
	seq[hg19]dup(22)(q12.1q13.33) chr22:g.25920001_51180000dup	25.26	Pathogenic	

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Table 2 (continued)

Case number	CNV-seq (hg19 chr:location)	Size, Mb	Clinical significance	Syndrome
18SC01837	seq[hg19]del(13)(q22.1q34) chr13:g.74840001_115100000del	40.26	Pathogenic	Microcoria - congenital nephrosis syndrome [OMIM #156600]
	seq[hg19]del(12)(q24.32) chr12:g.126500001_126660000del	0.16	VOUS	–
	seq[hg19]dup(6)(q24.1) chr6:g.140820001_141160000dup	0.34	VOUS	–
	seq[hg19]dup(16)(q22.1) chr16:g.69300001_69500000dup	0.2	VOUS	–
18SC38600	seq[hg19]del(17)(q12) chr17:g.34420000_36220000del	1.8	Pathogenic	Renal cysts and diabetes (RCAD) [OMIM #137920]
	seq[hg19]dup(X)(p22.31) chrX:g.8440000_8720000dup	0.28	VOUS	–
18SC41575	seq[hg19]del(7)(q33q36.3) chr7:g.136120001_159138663del	23.02	Pathogenic	Developmental retardation [OMIM 156600]; holoprosencephaly (HPE) [PMID:24550762]
	seq[hg19]dup(14)(q21.1q32.33) chr14:g.42580001_107300000dup	64.72	Pathogenic	Mental retardation [PMID:9268110] [PMID:18434272] [PMID:6851224]
19SC01153	seq[hg19]del(17)(q12) chr17:g.34800001_36260000del	1.46	Pathogenic	Renal cysts and diabetes (RCAD) [OMIM #137920]
	seq[hg19]dup(7)(p15.2) chr7:g.26160001_26440000dup	0.28	VOUS	–
	seq[hg19]dup(17)(p13.2) chr17:g.4620001_4900000dup	0.28	VOUS	–
19SC01182	seq[hg19]del(17)(p12) chr17:g.14540001_15260000del	0.72	Pathogenic	Hereditary liability to pressure palsies (HNPP) [OMIM #162500] [PMID:6851224] [PMID:24726093, 26982983] [PMID:10227632, 26982985]
19SC01200	seq[hg19]del(13)(q12.12) chr13:g.23540001_24940000del	1.40	Pathogenic	Spastic ataxia of the Charlevoix-Saguenay type (SACS) [OMIM *604490] [OMIM #270550] [PMID:18398442]/ combined oxidative phosphorylation deficiency-31 (COXPD31) [OMIM #617228]
	seq[hg19]dup(2)(q12.2q12.3) chr2:g.106880001_108440000dup	1.56	VOUS	–
19SC22446	seq[hg19]del(8)(p23.3p11.1) chr8:g.160001_43800000del	43.64	Pathogenic	8p23.1 deletion syndrome [OMIM #617228] [PMID:8533822]
	seq[hg19]dup(8)(q11.1q24.3) chr8:g.46880001_146300000dup	99.42	Pathogenic	Chromosome 8q22.1 duplication syndrome [OMIM #151200] [PMID:28419948] [PMID:20101682]
	seq[hg19]dup(13)(q14.3) chr13:g.54580001_55260000dup	0.68	VOUS	–

VOUS, variants of uncertain significance; OMIM, Online Mendelian Inheritance in Man; PMID, PubMed Unique Identifier; chr, chromosome; del, deletion; dup, duplication; Mb, megabases.

93.97%. Two and 5 samples could not be sequenced due to specimen decay and maternal component contamination, respectively. Of the 109 cases, 22 (20.18%) had normal results and 87 (79.82%) had abnormal results, of which 64 (58.72%) were numerical chromosome abnormalities (Table 1). Concerning numerical abnormalities of chromosomes, 2 cases were polyploid; 7 cases were chimeras, with the lowest abnormal karyotype proportion of 10%; 1 case was 70,XXY,+21. Ten of the 109 cases (9.17%) showed pathogenic CNVs (Table 2), and in 13 cases (11.93%) variants of uncertain significance (VOUS) were detected (Table 2). CNVs ≥10 Mb were defined as partial

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Table 3. Relationship between the size of CNVs and pathogenicity

Size, Mb	*N*	Pathogenic CNVs		VOUS	
		n	Proportion, %	*n*	Proportion, %
>5	5	5	100	0	0
1–5	4	4	100	0	0
0.1–1	14	1	7.14	13	92.86

aneuploidy, and CNVs <10 Mb were defined as microdeletion/microduplication. Pathogenic CNVs corresponded to the area where the specific microdeletion/-duplication syndrome was located and to the pathogenic fragment; CNVs >5 Mb were defined as possibly pathogenic.

A total of 50 out of the 64 cases of numerical chromosome abnormalities were single-chromosome aneuploidies, in which 47,XN,+22; 47,XN,+21; 47,XN,+16; and 47,XN,+13 were the most common (Fig. 1). There were 10 cases of chromosomal monosomy, 9 of which were 45,X combined with VOUS CNV and one was 45,XN,–21 combined with VOUS CNV. The double trisomy karyotypes in 4 cases were 46,X,+20; 48,XY,+4,+20 (combined with pathogenic seq[hg19]dup(9)(p24.3)chr9:g.300001_600000dup); 48,XY,+15,+16; and 48,XY,+14,+21. The chromosomal aneuploid chimera karyotypes in 7 cases were 47,XXX[10%]/46,XX[90%]; 47,XY,+22[30%]/46,XY[70%]; 47,XY,+21[65%]/46,XY[35%]; 47,XY,+21[50%]/46,XY[50%]; 47,XY,+21[35%]/46,XY[65%] combined with VOUS CNVs seq[hg19]dup(13)(q13.1), 47,XX,+16[80%]/46,X,+16[20%]; and 45,XY,–3[15%]/46,XY[85%]. The lowest abnormal karyotype ratio was 10%.

In 13 cases of VOUS and 10 cases of pathogenic CNVs, genome CNV fragments >1 Mb were clearly pathogenic aberrations. In 4 cases, genome CNV fragments of 0.1–1 Mb, except for one, were clearly pathogenic, and all others were VOUS. The detection system did not detect aberrations in fragments <0.1 Mb (Tables 2, 3). Pathogenic CNV sequencing results are shown in Figure 2.

The incidence of numerical chromosome abnormalities was significantly higher when pregnant women were older than 35 years and younger than 24 years, and it increased steadily in women older than 25 years of age (Fig. 1). Pathogenic CNVs and VOUS were not significantly different between the age groups.

The highest rate of numerical chromosome abnormalities was found in aborted embryos from 8 to 9 weeks and 6 days of gestation. Numerical chromosome abnormalities were determined in abortions before 13 weeks and 6 days of pregnancy. No numerical chromosome abnormalities, pathogenic CNVs, and VOUS were detected in fetuses at 14–15 weeks and 6 days of pregnancy (Fig. 1).

Discussion

Chromosome microarray analysis (CMA) is routinely used in clinical practice for pediatric and prenatal genetic diagnosis but rarely for abortion analysis. With the popularity of NGS in non-invasive prenatal testing, many community hospitals have established high-throughput gene sequencing platforms. CNV-seq and SNP arrays have a high degree of detection consistency in the identification of chromosomal diseases [Liang et al., 2014]. SNP-based CMA is a robust platform, with successful results obtained in >90% of cases. SNP-based CMA can identify aneuploidy, polyploidy, and whole-genome homozygosity, as well as segmental genomic imbalances and maternal cell contamination, thus maximizing sensitivity and reducing false-negative results. Besides SNP arrays, CNVs <5 Mb can also be identified by other array techniques. The additional value of SNP arrays is the allele information it provides on UPD and LOH. Thus, CNV-seq is expected to become a more scalable and affordable chromosomal disease detection technology than microarray.

In this study, CNV-seq was used to analyze 116 samples of aborted tissues in early and middle pregnancy. The success rate was 93.97% (109/116). There were 5 and 2 cases of unsuccessful results due to maternal blood contamination and DNA contamination, respectively. Upon embryo abortion, specimens should be retrieved as early as possible to prevent tissue decay. Of the 109 specimens successfully tested in this study, there were 87 cases (79.82%) with chromosomal aberrations, in which 74 cases (74/109, 67.89%) of pathological aberrations were identified; these findings are higher than those reported by several investigators [Levy et al., 2014; Sahoo et al., 2017; Dai et al., 2019; Zhang et al., 2019]. However, the sample size was small, and this might have biased the results. We could not identify balanced chromosomal rearrangements because CNV-seq cannot detect these rearrangements [Rosenfeld et al., 2015]. The limitation of NGS compared to traditional karyotyping is finding balanced translocations, but the limitation of NGS for CNV assessment, is the inability to detect copy-neutral events (LOH, UPD, female triploidy). No sex chromosome trisomy was

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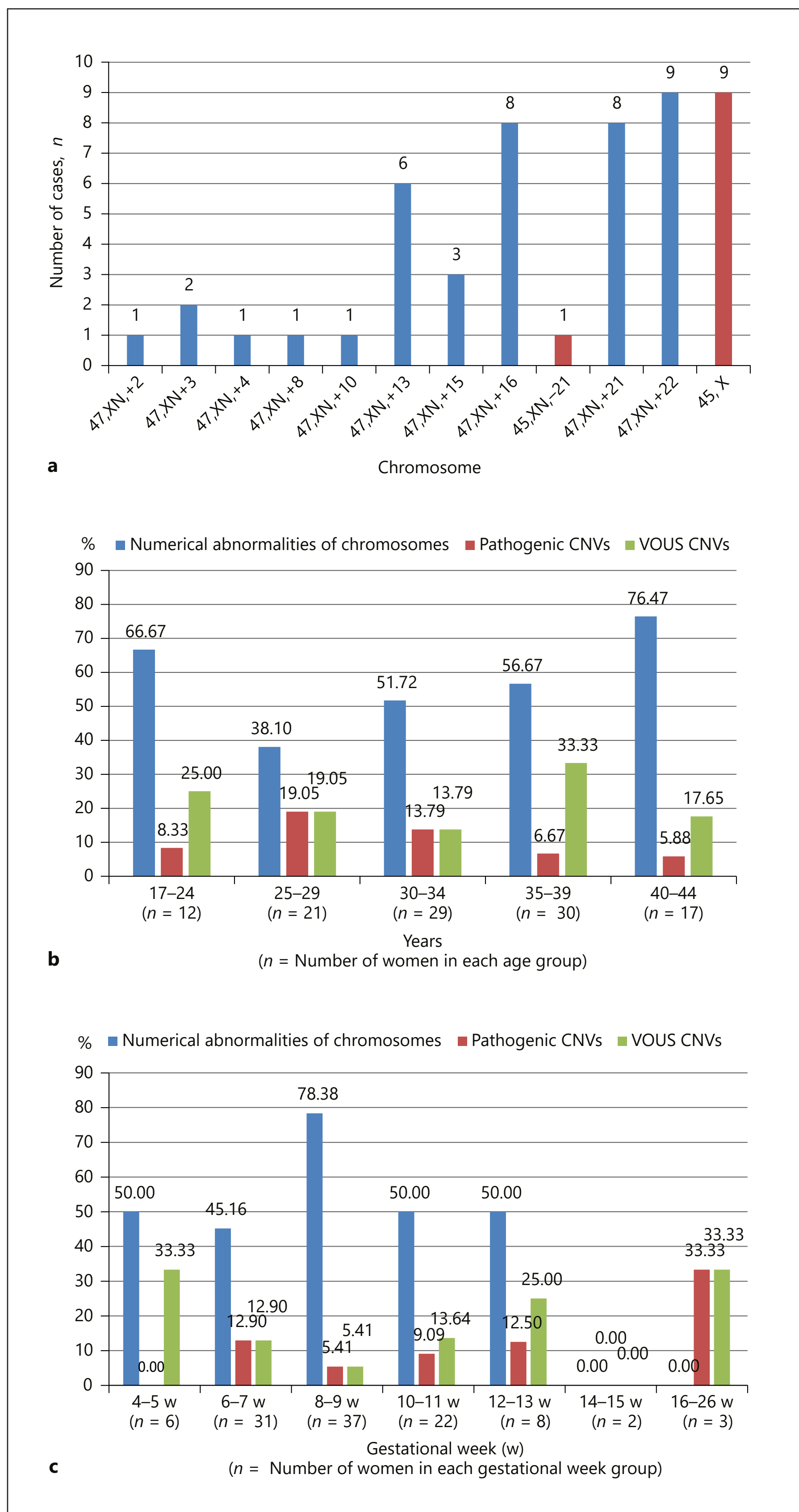

Fig. 1. Chromosomal variation analysis. **a** Incidence of single-chromosome aneuploidies (XN = XX or XY). **b** Incidence of numerical chromosome abnormalities, pathogenic CNVs, and VOUS depending on the maternal age. **c** Chromosome aberrations in abortion samples of different gestational weeks.

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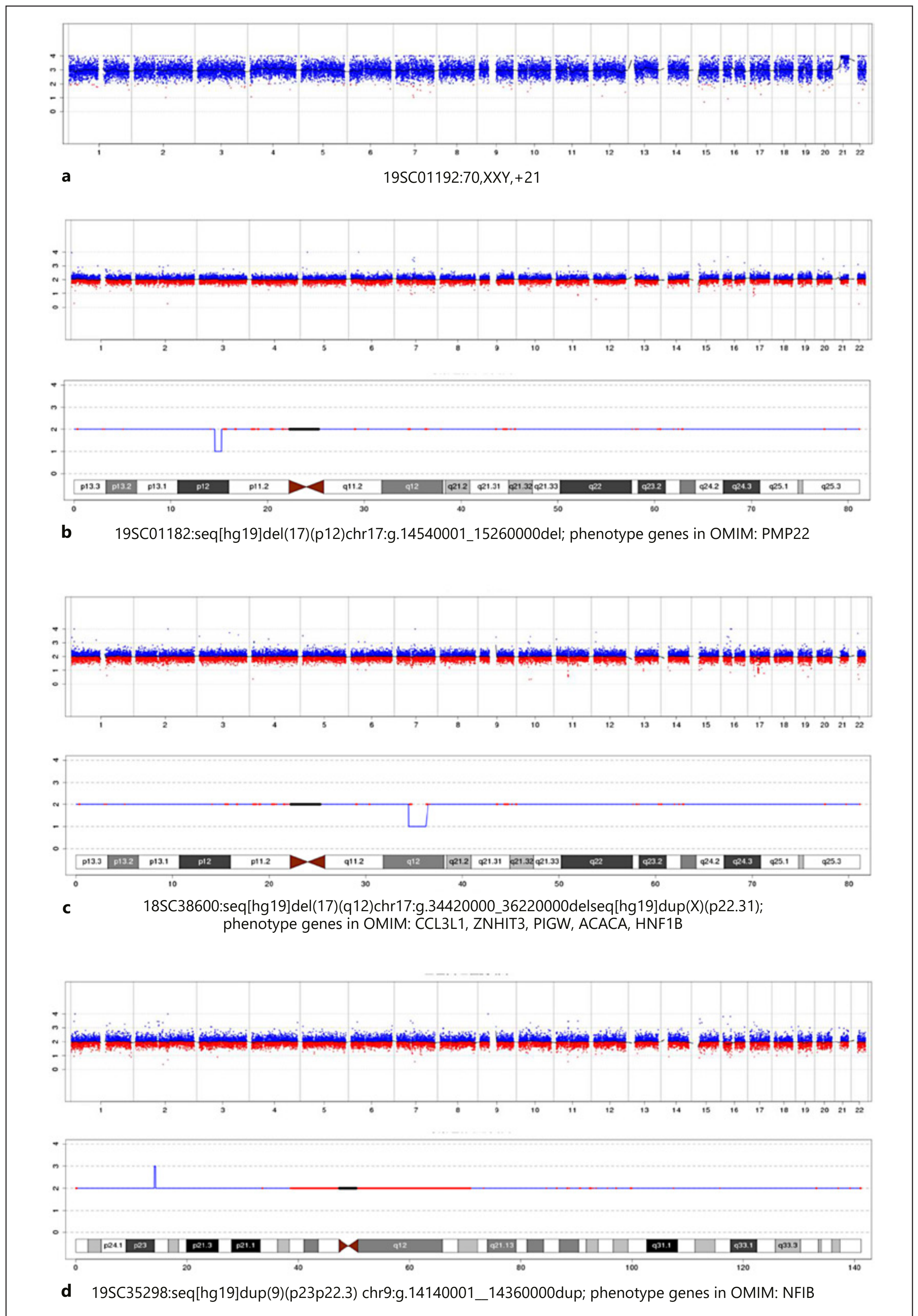

Fig. 2. Partial CNV-seq test results.

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identified in this study because it is less lethal; thus, it is barely detected in abortions. Sex chromosome monosomy is more common in abortions, and 45,X is the most common aneuploid karyotype. Autosomal monosomy is rarely seen in spontaneous abortions due to high mortality [Diego-Alvarez et al., 2006]. Only one case of 45,XN,–21 and one case of 45,XY,–3[15%]/46,XY[85%] were found in this study. In addition, 45,XN,–21 is also occasionally identified in prenatal diagnosis. It is speculated that 45,XN,–21 is less lethal than other autosomal monosomies and arises during the second trimester. However, there are no reported cases of 45,XN,–3 in prenatal diagnosis and live births. In this case, the fetus survived until the first trimester, which may have been due to the low proportion of abnormal cells with 45,XN,–3 karyotype. For the mosaic case with 45,XN,–3, it would be important to determine if the normal cell line shows hints of iUPD3. This can also be determined by sequencing data. In chromosomal double trisomy, one chromosomal trisomy usually involves an acrocentric chromosome. Abnormal CNV-seq signals are also highly sensitive and specific for suggesting chromosomal microdeletions/microduplications, and they can detect chromosomal microdeletions/microduplications that cannot be found by conventional karyotype analysis [Wang et al., 2017]. In addition, CNV-seq can detect chromosome chimerism aberrations. In our case, we could detect chimeras with a proportion of 10% abnormal cells. CNV-seq can not only accurately detect numerical abnormalities of chromosomes, but also CNVs. CNVs are the second leading cause of spontaneous abortion. In this study, 18 cases (18/109, 16.51%) with CNVs ≤5 Mb were detected (Table 3). Theoretically, these cases could not be detected by karyotype analysis. There was only one case of a pathogenic CNV fragment between 0.1 and 1 Mb, and it was identified in a 29-year-old woman at 9 weeks and 3 days of pregnancy. The CNV fragment was 0.72 Mb, covering approximately 52% of the hereditary liability to pressure palsies (HNPP) locus, and it contained the key gene responsible for the syndrome, *PMP22* [Wilke et al., 2000]. In addition, the age at onset of HNPP varies widely, and most patients have electrophysiological abnormalities [Luigetti et al., 2014].

Chromosomal abnormalities in aborted tissues are related to the maternal age. The incidence of numerical chromosome abnormalities in pregnant women older than 35 years of age and younger than 24 years of age was significantly higher. In women older than 40 years of age, the detection rate of numerical chromosome abnormalities was even as high as 76.47%. Furthermore, the incidence of fetal pathogenic CNVs in pregnant women between 25 and 29 years of age was significantly higher. It has been reported that 80% of miscarriages occur in the first 12 weeks of pregnancy [Yuen et al., 1981], and they are rare in the second trimester of pregnancy [Sánchez et al., 1999]. No numerical chromosome abnormalities were identified in aborted embryos at 14 weeks of gestation or older, but the incidence was very high at 8–9 weeks, indicating that numerical chromosome abnormalities are an important cause of early embryo loss. The detection system could not identify aberrations in fragments smaller than 0.1 Mb. With the continuous updating of open genetic databases, an increasing number of clinically significant micro-imbalances will be identified.

Unfortunately, the parents of our cases were not tested. Regardless, in cases of VOUS or recurrent microdeletions/duplications, it is better to understand the clinical impact and to calculate the recurrence risk for subsequent pregnancies. Spontaneous abortion is a natural process of the human body to eliminate embryos with chromosomal abnormalities. Defining the cause of miscarriage can reduce the psychological burden of patients and provide a reliable basis for the patients' reproductive guidance. CNV-seq can not only accurately detect numerical chromosome abnormalities, but also CNVs that cannot be identified by conventional karyotype analysis. CNV-seq is of great significance in the determination of the cause of abortion.

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Statement of Ethics

The patients had provided their informed consent for publication. This study is retrospective and did not require ethical approval.

Conflict of Interest Statement

The authors declare that they have no competing interests.

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Author Contributions

S.L. designed the study, performed experimental studies, and drafted the manuscript. X.C. compiled the data, contributed to writing, and helped in drafting the manuscript. J.Y. and Z.X. performed analyses and helped in drafting the manuscript. P.C. and D.Y. reviewed the manuscript and helped in shaping the manuscript. T.Y. and N.T. performed analyses and provided valuable suggestions and clinical outputs. All authors read and approved the final manuscript.

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