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Identification of a complex chromosomal insertion using the chromosome conformation based karyotyping technique for the implementation of PGT-SR

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Abstract

Objective This study completed the karyotyping of a patient with a complex chromosomal insertion and identified the location of the breakpoint for implementing preimplantation genetic testing for chromosomal structural rearrangements (PGT-SR) to differentiate between normal and carrier embryos, aiming to assess the clinical outcome of PGT-SR in couples with complex chromosome rearrangements (CCRs).

Method The Chromosome conformation based Karyotyping technique (C-Moka) was implemented to identify karyotypes and analyze chromosomal breakpoints, with subsequent verification of karyotype results by fluorescence in situ hybridization (FISH). Whole genome sequencing (WGS) of embryos followed by copy number variation and second-generation sequencing (NGS) based single nucleotide polymorphism (SNP) haplotyping to discriminate between normal and carrier embryos were carried out in PGT-SR cycles.

Results Based on the precise breakpoint sequences identified by C-Moka, mapping allele with resolved carrier status (MaReCs) was used to distinguish a normal embryo from a carrier embryo among two balanced euploidy embryos, resulting in the birth of a healthy baby after transfer of the normal embryo.

Conclusion This case demonstrates the feasibility of C-Moka technique in assisting CCRs diagnosis and directly identifying breakpoints to construct haplotypes without family lineage or reference embryo pre-tests.

Keywords Complex chromosomal rearrangements, Preimplantation genetic testing for chromosomal structural rearrangements, Chromosome conformation-based karyotyping, Mapping allele with resolved carrier status, Complex chromosomal insertion

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What's already known about this topic?

Currently, known techniques for identifying breakpoint regions of CCRs during PGT-SR are too limited to distinguish carriers from karyotypic normal embryos.

What does this study add?

The Chromosome conformation based karyotyping technique (C-Moka) was used to identify the chromosomal karyotype of a patient with missed CCRs and provide precise breakpoint sequences to differentiate normal embryos from two balanced euploid embryos. The C-Moka technique can assist in diagnosing complex chromosomal rearrangements, directly identify breakpoints, and construct haplotypes.

Background

Complex chromosomal rearrangements (CCRs) are structural abnormalities characterized by two or more chromosomes with three or more cytogenetic breakpoints, resulting in the exchange of genetic material between non-homologous chromosomes [1]. CCRs can be balanced or unbalanced that is, absence or presence of chromosomal copy number alterations. CCRs are also rare with about 260 cases reported worldwide [2]. Clinical evaluation of chromosomal abnormalities by traditional cytogenetic techniques is unable to detect submicroscopic rearrangements, and rearrangement fragments of similar size and the same banding patterns do not readily identify translocations, and the true incidence of chromosomal translocations/inversions is likely to be much higher than the actual detection rate [3]. Due to the resolution (with a detection limit ≥ 5–10 Mb), traditional cytogenetic techniques have difficulty in detecting these breakpoint regions, and only by relying on molecular cytogenetic techniques can the complexity of CCRs be clearly characterized. For a long time, fluorescence in situ hybridization (FISH), chromosome microarray analysis (CMA), copy number variation sequencing (CNV-Seq), whole genome sequencing (WGS), and chromosome sequencing have all been gradually applied to CCRs detection, and different detection technologies have different advantages and limitations [4, 5].

The chromosome conformation capture method is builds upon the"proximity ligation"concept introduced in the Nuclear Ligation Assay [6]. In this approach, chromatin is cross-linked with formaldehyde, digested with restriction enzymes, and then subjected to proximity ligation, capturing spatially adjacent chromatin fragments. The resulting ligation products are detected via site-specific PCR [7]. Based on this foundation, we introduce a novel molecular karyotyping technology termed Chromosome Conformation Based Karyotyping Technique (C-Moka). This method utilizes whole cell nuclei as the starting material and combines high-throughput sequencing with bioinformatic analyses to investigate genome-wide chromatin spatial relationships. By capturing genome-wide DNA interaction patterns, C-Moka provides high-resolution three-dimensional structural information on chromatin architecture. It leverages the principle that physically proximate genomic regions are more likely to interact with each other, thereby enabling the detection of structural variants (SVs) by capturing abnormal or unexpected interactions between distant genomic loci. Through special bioinformatics algorithms, it enables accurate and comprehensive detection of balanced and unbalanced translocations, insertions, deletions, inversions and other complex or cryptic chromosomal structural abnormalities [8]. Additionally, the technique supports mapping allele with resolved carrier status (MaReCs) [9], facilitating the distinction between normal and carrier embryos in cases of chromosomal structural variations and CCRs. This is achieved through breakpoint confirmation and haplotype construction without requiring familial reference samples or pre-test embryos.

To demonstrate the clinical utility of C-Moka, we present a case involving a female carrying CCRs comprising five breakpoints across three chromosomes. Initial classical cytogenetic analysis using G-banding (550-band resolution) suggested a karyotype of 46,XX, ins(16;18)(q23;q11.2q12.3). However, preimplantation genetic testing for structural rearrangements (PGT-SR) results from 20 embryos over three IVF cycles indicated three breakpoints on chromosome 18, raising suspicion of an undetected CCR. Subsequent analysis using C-Moka confirmed a more complex karyotype: 46,XX, ins(5;18)(q21.3;q21.1q12.3),ins(16;18)(q23.1;q11.2q12.3). The MaReCs technique was utilized to discriminate and select a cytogenetically normal embryo from euploid carriers, resulting in the successful transfer and subsequent achievement of a full-term healthy live birth. This case highlights the capability of C-Moka to facilitate accurate diagnosis of CCRs and enable breakpoint resolution and haplotype construction in the absence of familial genetic data or reference embryo testing.

Materials and methods

Patients

The proband (Fig. 1A) was a 31-year-old female who had a history of induced abortion during the second trimester due to a chromosomal duplication of approximately 10 Mb at 18q11.2q12.3 in the fetus. She was subsequently referred to the Reproduction and Genetics Center of Yichang Central People's Hospital for PGT-SR due to fertility problems. Classical cytogenetic examination revealed karyotype of the female was established as 46,XX, ins(16;18)(q23;q11.2q12.3) by G-banding resolution of 550 (Fig. 1C) with CNV-seq suggesting euploidy

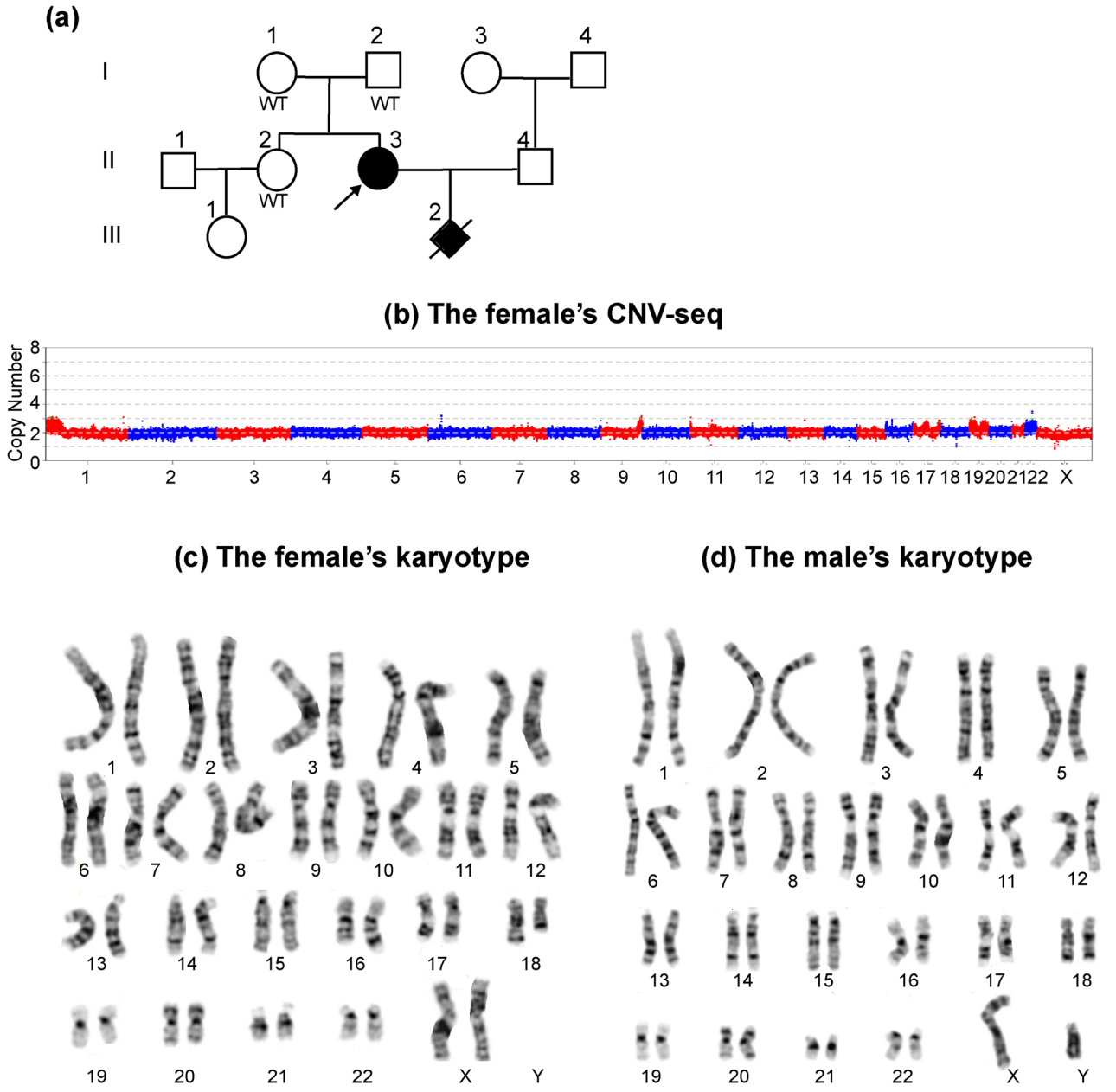


Fig. 1 Results of cytogenetic and molecular genetic testing of chromosome insertion family lines. **A**. Patient's pedigree, showing the carriers of the complex chromosome translocation in the family (black circle) and miscarriages (black parallelogram). Arrow indicates proband. **B**. CNV-seq showed that the female was euploid. **C**. G-banded karyotype analysis at a band resolution of 550 of the female, 46,XX, ins(16;18)(q23;q11.2q12.3). **D**. The male's karyotype is 46,XY

(Fig. 1B). Karyotypes of female's husband was normal (Fig. 1D).

NGS-based preimplantation genetic testing for structural rearrangement

Three PGT-SR cycles of controlled ovarian hyper-stimulation, aspiration of the follicles, Intracytoplasmic sperm injection (ICSI), embryo culture, blastocysts trophectoderm biopsy, sample collection, and embryo transfer were carried out at the Reproduction and Genetics Center of Yichang Central People's Hospital. The first 2 cycles of whole genome amplification (WGA), library preparation, next-generation sequencing (NGS) and data analysis were performed at Peking Jabrehoo Med Tech Co., Ltd, and the last 1 cycle was performed at Suzhou Yikon Genomics. Multiple annealing and looping-based amplification cycles (MALBAC) (Yikon Genomics, Soochow, China) were applied for WGA. The WGA product was used for copy number variation (CNV) library preparation via an NGS library preparation kit (Yikon Genomics). Library

sequencing was performed using the Nextseq CN500 instrument (Illumina, San Diego, CA, United States). For CNV analysis, sequencing files were disposed in the ChromGo (Yikon Genomics) software. High-throughput sequencing results of 20 biopsies of human early blastocyst trophoblast cells (SUB15415567) used in this study was downloaded from BioSample database (https://submit.ncbi.nlm.nih.gov/subs/sra/SUB15415567/overview) at the National Center for Biotechnology Information. A transferable embryo was defined as a balanced euploid embryo. Patients were informed that there was a 50% chance that the infant karyotype would either be a normal euploid or a balanced carrier euploid.

The chromosome conformation based karyotyping technique (C-Moka)

Chromosome conformation capture was performed as described previously with some modifications [10–12]. The C-MoKa chromosome detection technology primarily involves the formaldehyde fixation of Peripheral Blood Mononuclear Cells (PBMCs), capture and enrichment of labeled sequences, library construction, DNA sequencing, and bioinformatic analysis. The workflow of the C-MoKa technology illustrated in Fig. 2. The detailed experimental procedure is as follows: Peripheral blood (2 mL) was collected into a single-use cell-free DNA preservation tube (Cap Color: Yellow; Catalog No.: CWY036M). PBMCs were isolated from the whole blood by centrifugation at 500 × g for 5 min. The red supernatant was discarded, and the cell pellet was washed and resuspended twice in an appropriate buffer to obtain a pure cell suspension. The PBMCs were then fixed using a 1% final concentration of formaldehyde (from a 37% stock solution) for crosslinking. Fixed cells were lysed using the Lysis Buffer from the Nucleic Acid Extraction and Purification Kit (Yikon Genomics Ltd., Catalog No. XK-064). Genomic DNA was concurrently digested using the Fragmentation Enzyme (DNase I) provided in the same kit (XK-064). The digested DNA was purified using magnetic beads (from the same kit, XK-064) to remove enzymes and other contaminants. The ends of the purified, digested DNA fragments were labeled and ligated using T4 DNA Ligase (New England Biolabs, Catalog No. M0202V). This step is crucial for subsequent circularization or specific end-joining. Formaldehyde (FA) crosslinking was reversed by incubating the samples in a solution containing 0.3% SDS and Proteinase K (New England Biolabs, Catalog No. P8107S) for 1 h at 60 °C.The DNA was then purified using AMPure XP beads (Beckman Coulter, Catalog No. A63881) to remove proteins and enzymes. The concentration of the purified DNA was quantified using a Qubit fluorometer, ensuring a minimum concentration of 5 ng/μL before proceeding. To achieve a optimal fragment size distribution for sequencing, the DNA was further sheared into fragments ranging from 200 to 600 bp using the DNA Fragmentation Kit (Yikon Genomics Ltd., Catalog No. KT110700324). DNA fragments within the desired size

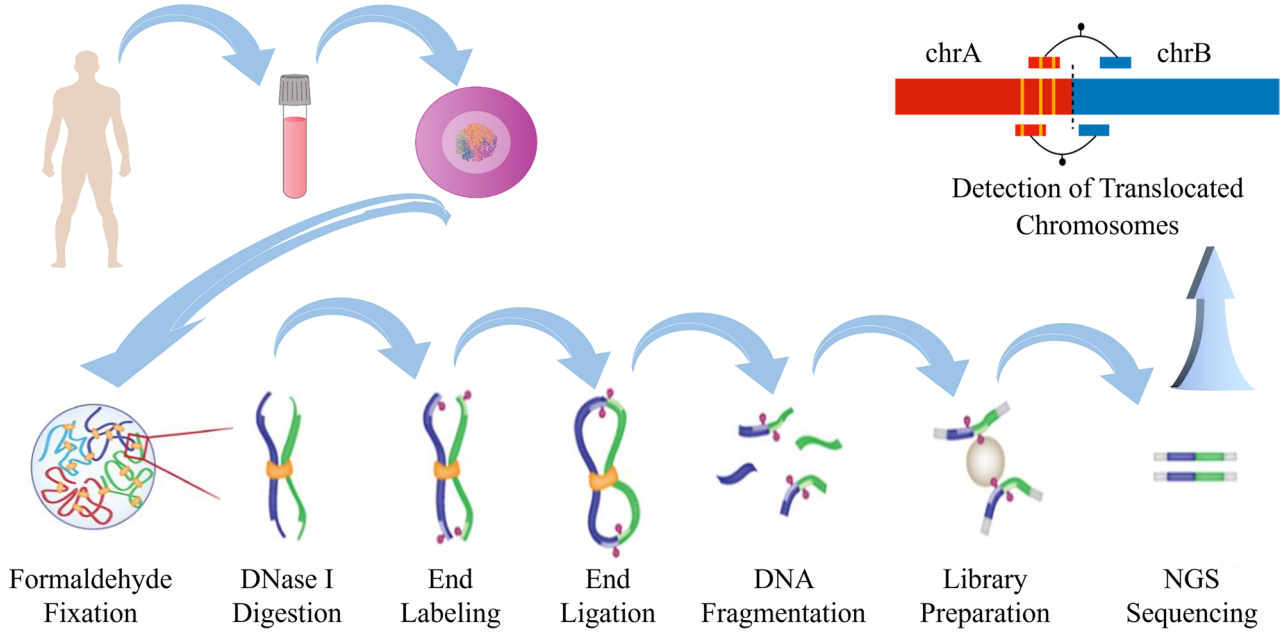


Fig. 2 Workflow of the C-MoKa Technology. This schematic illustrates the streamlined workflow for detecting chromosomal translocations. Isolated PBMCs are formaldehyde-fixed to preserve nuclear structures. Genomic DNA is then fragmented by DNase I digestion, followed by end labeling and ligation. After crosslink reversal and purification, DNA is sheared to optimal size for library construction, which includes end repair, adapter ligation, and PCR amplification. Finally, libraries are sequenced on a high-throughput platform, and bioinformatic analysis identifies translocated chromosomes

range were selected using AMPure XP beads with specific bead-to-sample ratios. End repair, dA-tailing, and adapter ligation were performed on the size-selected DNA fragments using the Library Preparation Kit (Yikon Genomics Ltd., Catalog No. KT100804048). Specifically, adapters from the Library Construction Adapter Primer Kit (Illumina compatible; Yikon Genomics Ltd., Catalog No. KT100806424) were used for ligation. Library amplification was carried out using the Amplification Mix from kit XK-048 and specific index primers from kit YK009 (Yikon Genomics Ltd.) for multiplexing. The final constructed libraries were subjected to quality control checks, including assessment of concentration, fragment size distribution, and absence of adapter dimers. High-throughput next-generation sequencing was performed using a DNBSEQ-T7 sequencer (MGI). Raw sequencing data underwent quality control checks using tools FastQC. High-quality paired-end reads were aligned to the appropriate reference human genome using optimized aligners BWA-MEM. SVs and CNVs were identified and statistically analyzed using specialized detection algorithms [8]. Detected variants were annotated using public and internal databases to determine their potential functional impact and clinical relevance. Further filtering and analysis were performed based on quality scores, population frequency, and predicted pathogenicity to prioritize significant findings. Hi-C is a widely employed method for studying how genomes are folded in three dimensions [11]. To interrogate all loci at once, the C-MoKa generates heatmaps of all-versus-all chromosome spatial interactions obtained through the Hi-C, which combines DNA proximity ligation with high-throughput sequencing in a genome-wide fashion [8].

Mapping allele with resolved carrier status (MaReCs)

To identify embryos that had not inherited the translocation, a strategy combining breakpoint mapping and haplotype analysis was employed. In accordance with the reported MaReCs methodology [9], the process began with MALBAC-based whole-genome amplification of TE biopsies, followed by NGS library preparation and sequencing for CNV and chromosomal copy number status assessment. After the precise breakpoint location was completed using the C-MoKa technology, linkage analysis was performed utilizing informative SNPs residing within 1 Mb on either side. This facilitated the construction of SNP-based haplotypes and the discrimination of alleles carrying the translocation, which together guided the final embryo selection.

Fluorescence *in situ* hybridization procedures (FISH)

The FISH assays were used to detect all chromosome segments involved in chromosomal rearrangement. FISH analysis was performed for the female to further verify the C-Moka results. The procedure was performed in metaphase in line with the manufacturer's instructions. Vysis CEP 16 Spectrum White (Abbott, Chicago, IL, United States), WCP 18 Green (Abbott), Tel Vysion 18p Spectrum Green (Abbott), Tel Vysion 18q Spectrum Red (Abbott), Tel Vysion 5p Spectrum Green (Abbott) and Tel Vysion 5q Spectrum Red (Abbott) probes were used for the metaphase FISH analysis. All operations followed the manufacturer's protocols.

Results

Clinical and embryological outcomes of PGT-SR cycles

Three PGT-SR cycles were carried out to selecting euploid embryos for transfer by analyzing chromosome imbalance and aneuploidy using NGS. A total of 40 oocytes was retrieved, including 37 mature oocytes, and 33 oocytes were observed as two pronuclear embryos after ICSI. In all, 20 blastocysts reached sufficient quality to undergo blastocysts trophectoderm biopsy (Table 1). In the first PGT-SR cycle, among the five blastocysts formed, Embryos 3 and 4 exhibited whole-chromosome deletions or duplications; Embryos 1, 2, and 4 showed deletions or duplications on chromosome 18; and Embryo 5 carried multiple chromosomal and segmental imbalances. No euploid embryo was available for transfer in this cycle (Figs. 1, 2, 3E1-E5, 4 and 5). In the second PGT-SR cycle, seven blastocysts were analyzed. Embryos 7, 8, 10, 11, and 12 carried deletions or duplications on chromosome 18 and/or other chromosomal abnormalities. Embryo 9 was detected with monosomy 16, a segmental deletion on chromosome 8, and multiple chromosomal mosaicisms. Only Embryo 6 was identified as euploid (Fig. 3, E6–E12). Therefore, the patient opted for direct frozen embryo transfer without further determining whether the euploid embryo was a carrier. Unfortunately, the transfer resulted in implantation failure. In the third PGT-SR cycle, eight blastocysts were formed. Embryos 14, 15, 16, 18, 19, and 20 showed deletions on chromosome 18 at regions 18q11.2, 18q12.3, and 18q21.1; in addition, Embryo 18 also showed an additional trisomy 20. Two balanced euploid embryos—Embryo 13 and Embryo 17—were identified in this cycle (Fig. 3, E13–E20).

Karyotyping and determination of the location of the breakpoints to differentiate normal embryos from carrier embryos with C-Moka technique

Summarizing the PGT-SR results from a total of 20 embryos across three IVF cycles suggested the presence of breakpoints at 18q11.2, 18q12.3, and 18q21.1 (as detailed in Table 1). This finding led to hypothesis that the female partner might harbor CCRs, which had not been detected by prior high-resolution karyotyping. To identify the breakpoints of the current rearrangement,

Table 1 Next-generation sequencing (NGS) outcome of three PGT-SR cycles

Cycle	Embryo No.	Biopsy day and grade	NGS
1	1	D5-5AB	46,XN, dup(18) (q11.2-q12.3) (16.20 Mb)
	2	D5-4BB	46,XN, del (18) (q12.3-q21.1) (4.80 Mb)
	3	D5-4BC	45,XN,-22
	4	D5-4BC	47,XN,+19,dup(18) (q11.2-q12.3) (16.20 Mb)
	5	D6-4CB	-2;-7;+8;+12;+13;-14;+19;-20;+21,del(1) (p31.2-q21.2) (77.88 Mb); dup(9) (p24.3-p13.1) (39.00 Mb); trip(9) (q13-q34.3) (74.70 Mb); del(11) (p15.5-p12) (40.75 Mb)
2	6	D5-4BA	46,XN
	7	D5-4AB	45,X, del(18) (q12.3-q21.1) (4.20 Mb)
	8	D5-4BB	46,XN,-(mosaic)15(54%),dup(mosaic) (1) (p35.2-p11.2) (90.07 Mb) (53%);del(18) (q12.3-q21.1) (4.20 Mb)
	9	D5-4BC	45,XN,-16;-(mosaic)17(55%);+(mosaic)22(47%),dup(8) (p22-p12) (16.80 Mb); dup(mosaic) (8) (p12-q24.3) (112.56 Mb) (44%)
	10	D5-4BC	46,XN, del(mosaic) (17) (q12-q25.3) (48.13 Mb) (65%);dup(18) (q11.2-q12.3) (15.60 Mb)
	11	D5-4BC	46,XN,-(mosaic)7(31%) ;-(mosaic)15(32%),del(mosaic) (2) (q21.3-q37.3) (107.65 Mb), (49%);del(18) (q11.2-q12.3) (15.60 Mb)
	12	D5-4CB	46,XN, dup(18) (q12.3-q21.1) (4.80 Mb)
3	13	D5-4BA	46,XN
	14	D5-4BA	46,XN, del(18) (q11.2q21.1) (~20.40 Mb)
	15	D5-4BB	46,XN, del(18) (q11.2q12.3) (~16.00 Mb)
	16	D5-4BB	46,XN, del(18) (q12.3q21.1) (~4.80 Mb)
	17	D5-4BB	46,XN
	18	D5-4BC	47,XN,+10,del(18) (q12.3q21.1) (~5.00 Mb)
	19	D6-4BB	46,XN, del(18) (q11.2q21.1) (~20.20 Mb)
	20	D6-4CB	46,XN, del(18) (q12.3q21.1) (~4.60 Mb)

we used the C-Moka technology strategy based on high-throughput sequencing and chromatin three-dimensional structural information of the nucleus. Subsequently, we performed the C-Moka technique to discover an inverted insertion of the q21.1q12.3 segment of chromosome 18 to chromosome 5 (Fig. 4, black circle), and an insertion of the q11.2q12.3 segment of chromosome 18 to chromosome 16 (Fig. 4, green circle), and also identified the locations of the five breakpoints in the three chromosomes involved (Fig. 5A), which was subsequently verified by FISH during the division phase (Fig. 5B). The karyotype of the female with complex chromosome insertion rearrangement was identified by C-Moka technology as 46,XX, ins(5;18)(q21.3;q21.1q12.3),ins(16;18)(q23.1;q11.2q12.3). Based on the precise breakpoint sequences provided by C-Moka technique, the MaReCs technique was used to distinguish the normal embryo 17 and the carrier embryo 13 from the two balanced euploid embryos (Fig. 6), the normal embryo 17 was transferred and the woman ultimately achieved a successful live birth.

Discussion

In this study, we conducted karyotype analysis of a patient with complex chromosome insertions via C-Moka and determined the breakpoints of PGT-SR to discriminate normal embryos from carrier embryos, thereby evaluating the clinical efficacy of the combination of C-Moka and PGT-SR in couples with CCRs.

Analysis of structural rearrangement information from the established WGS database of the 1000 Human Genomes Project identified four balanced translocations and four inversions with an incidence of 1 in 291.5 (0.34%, 4/1166), and the estimated rate of balanced translocations from karyotypic analysis of G-banded chromosomes was 0.16–0.2% [13]. Of the 126 couples (39.7% 50/126) who had undergone routine karyotyping to confirm a normal karyotype, additional chromosomal abnormalities were detected by genome sequencing, including 8 balanced translocations and 42 inversions, suggesting that hidden structural chromosomal abnormalities undetectable by karyotyping are much higher than expected [14]. In this study, classical cytogenetic techniques identified structural recombination only in large portions of chromosomes 18 and 16, leading to misjudgment of the female's probability of obtaining a normal embryo. It has been reported that the odds of balanced or normal embryos in couples with CCRs are < 6% [15]. Therefore, recurrent spontaneous abortion, arrested intrauterine pregnancy, fetal malformation and infertility often happen. In this study, as many as 70% (14/20) of the embryos of the CCRs female were imbalanced due to chromosomal insertions. So PGT-SR is performed before embryo transfer, and a small portion of cells will be aspirated for comprehensive chromosome screening to analyze

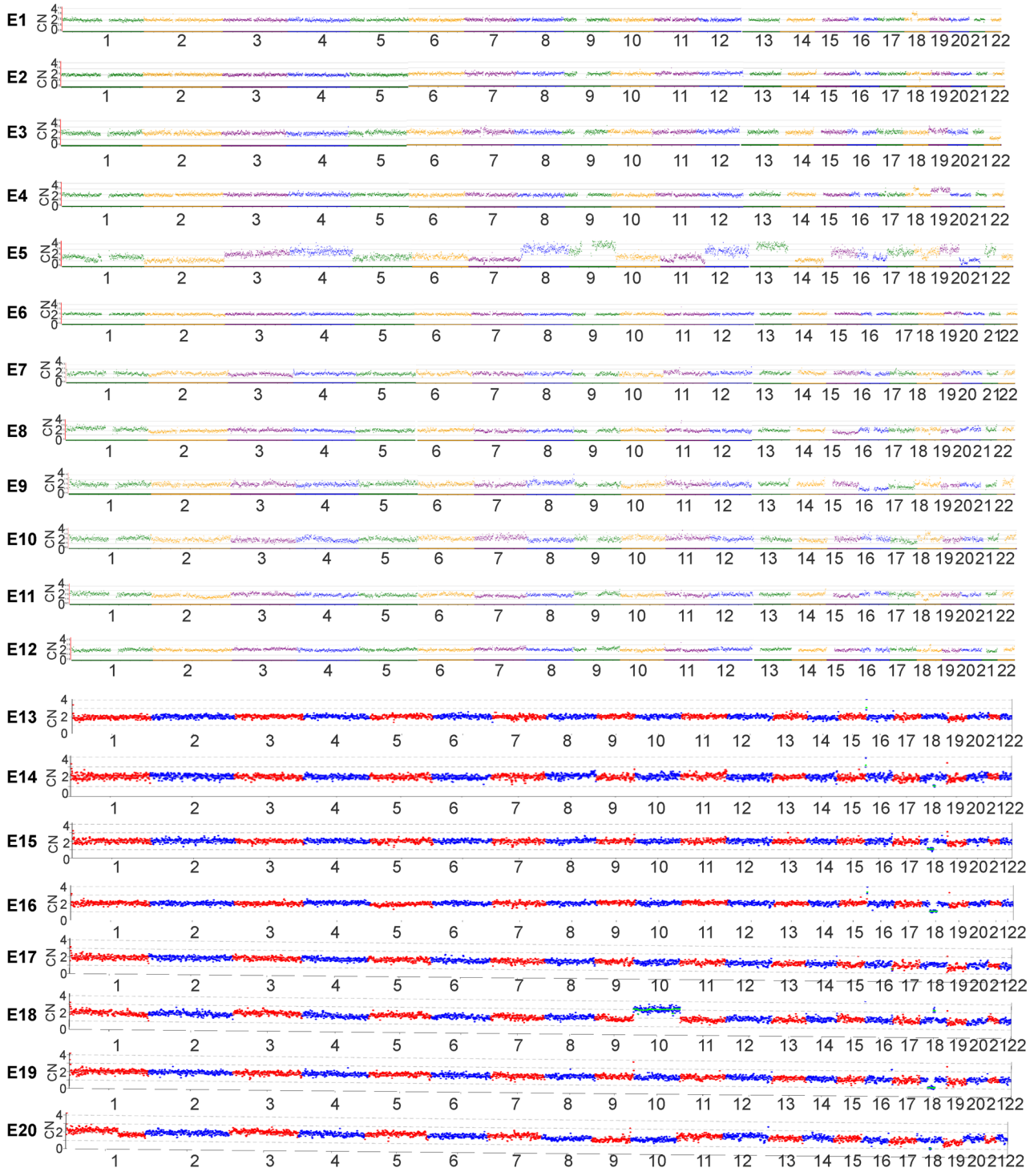


Fig. 3 Copy number variation (CNV) results of twenty embryos across three PGT-SR cycles. **E1-E5**: First cycle (Embryos 1–5): Embryos 1, 2, and 4 showed deletions/duplications at 18q11.2, 18q12.3, and 18q21.1; Embryo 4 also presented trisomy 19; Embryo 3 had monosomy 22. **E6-E12**: Second cycle (Embryos 6–12). Embryos 7, 8, 10, 11, and 12 carried deletions/duplications at 18q11.2, 18q12.3, and 18q21.1 and/or other abnormalities; Embryo 9 exhibited monosomy 16, a segmental deletion on chromosome 8, and mosaicism; only Embryo 6 was euploid. **E13-E20**: Third cycle (Embryos 13–20). Embryos 14, 15, 16, 18, 19, and 20 showed deletions at 18q11.2, 18q12.3, and 18q21.1; Embryo 18 also showed trisomy 20. Only Embryos 13 and 17 were identified as euploid. E, embryo; CN, copy number

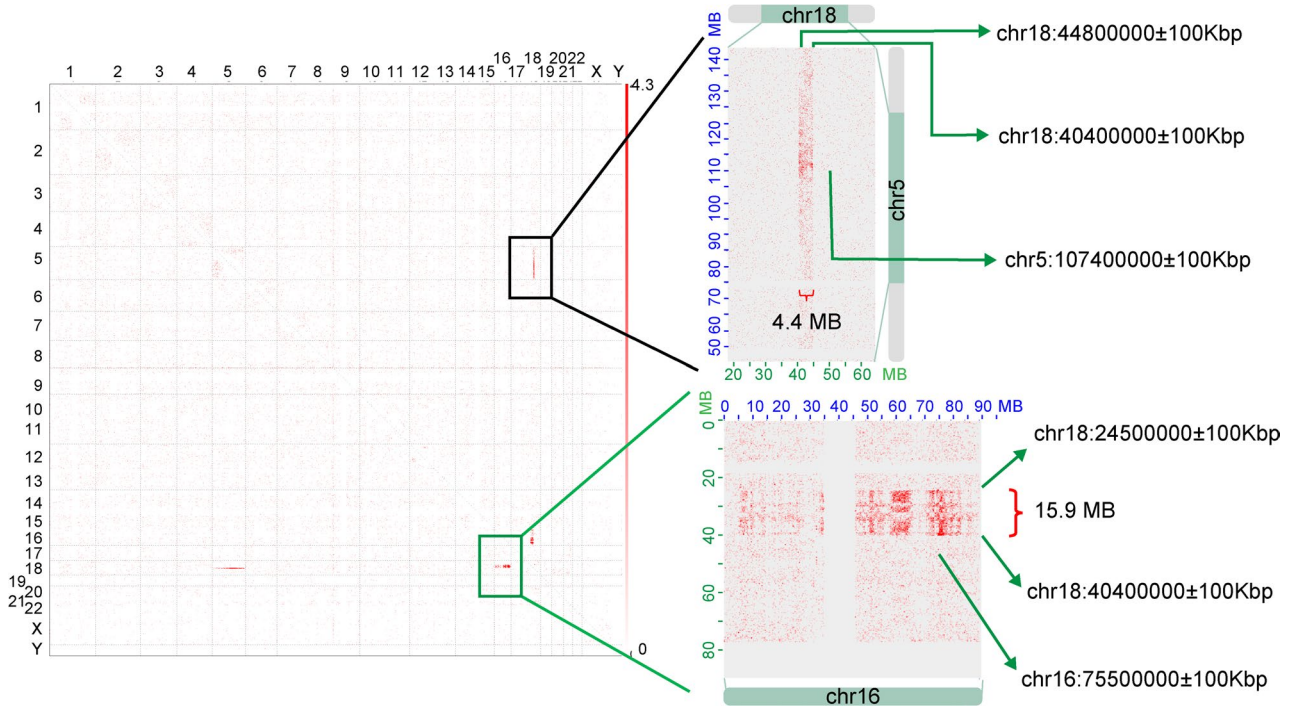
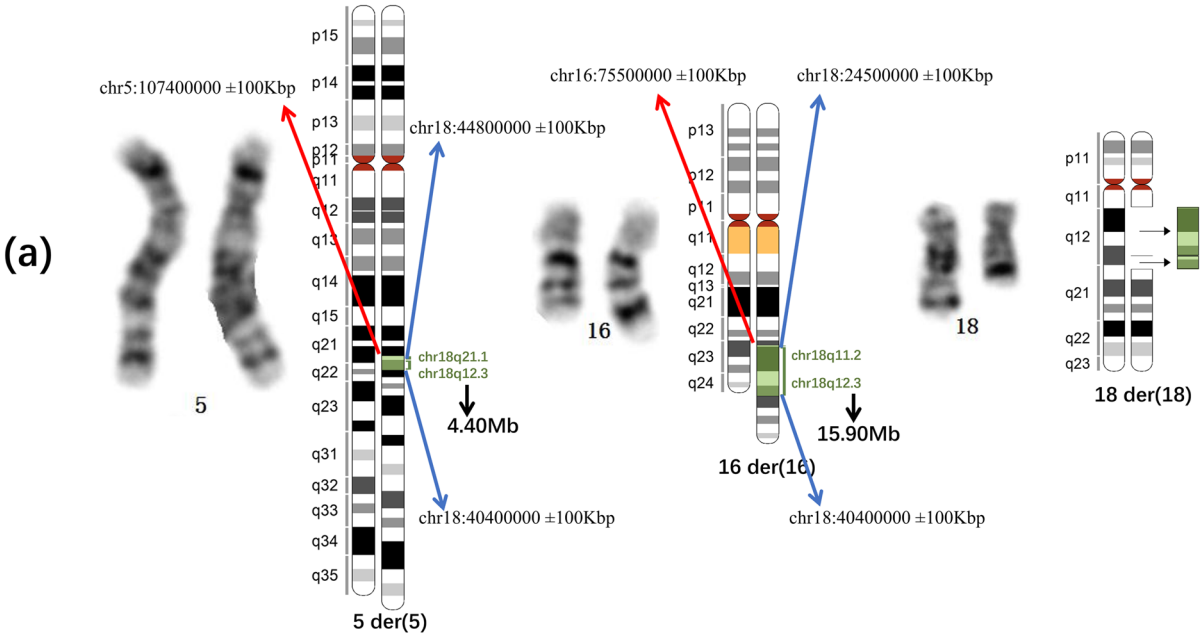


Fig. 4 The C-MoKa revealed the following complex insertion events in female peripheral blood chromosome samples via Hi-C contact maps: ins(5;18)(q21.3;q21.1q12.3) and ins(16;18)(q23.1;q11.2q12.3). Both the X and Y axes correspond to chromosomal positions binned at a fixed resolution. Each pixel reflects the interaction frequency between two genomic loci, with darker colors indicating stronger spatial proximity. Fill length ratio is proportional to chromosome size and fill color is proportional to chromosome density. C-Moka analysis identified two complex insertions: an inverted insertion of a 4.4 Mb segment from chromosome 18 into chromosome 5 (black circle), and a 15.9 Mb insertion from chromosome 18 into chromosome 16 (green circle). Black arrows mark breakpoint locations. These aberrant interaction signals indicate structural variations that bring distal genomic regions into spatial proximity

embryos identified as balanced or normal for transplantation [16]. The method can reduce the miscarriage rate and improve clinical outcomes [17]. However, the probability of obtaining normal or balanced embryos in CCRs carriers is very low, as demonstrated by our finding that only 15% (3/20) of the CCRs female had balanced embryos.

C-Moka based on high-throughput sequencing can reveal the spatial conformation of chromatin within the nucleus [18] and demonstrated that it is organized hierarchically in chromosome territories [19], chromosome compartments [20], topological associated domains (TADs) [21], and DNA loops [22]. In this study, C-Moka demonstrates its great advantage in detecting CCRs or in patients with questionable karyotyping. Its ability to accurately and comprehensively localize structural rearrangements can effectively assist in the clinical diagnosis of CCRs [23], especially in cryptic balanced translocations and inversions and CCRs involving multiple chromosomes [24]. Conventional cytogenetic analysis has a limited resolution (typically 5–10 Mb) and is therefore prone to miss small chromosomal segments and breakpoints. Furthermore, its accuracy is highly dependent on the analyst's expertise. The experimental procedure is complicated and mostly manual, which makes it difficult to be standardized [5]. In this case, conventional karyotype of the female suggested that only autosomes 16 and 18 were rearranged, but C-Moka detected the involvement of a third chromosome (chromosome 5), revealing a complex rearrangement involving three chromosomes. Although the conventional karyotype results suspected a fragment of chromosome 18 was inserted into the chromosome 16, C-Moka accurately detected that it was divided into two parts: a 15.90 Mb part inserted into chromosome 16 and a 4.40 Mb part inverted and inserted into chromosome 5. C-Moka can detect chromosomal structural abnormalities below 5 Mb with high resolution. Unlike manual karyotype analysis by visual observation, C-Moka enables accurate detection of balanced translocations with high resolution. It achieves this by leveraging high-throughput sequencing and bioinformatic automation to capture three-dimensional chromatin structure, and by analyzing breakpoint signals and sub-chromosomal (arm-level) variants to detect translocations in cryptic regions [25]. Since there is no need for cell culture and no need to consider the cell growth status, C-Moka has a promising application in the field of



Breakpoint position:

chr5:107400000 ±100kbp, chr16:75500000 ±100kbp, chr18:24500000 ±100kbp, chr18:40400000 ±100kbp, chr18:44800000 ±100kbp

Chromosome linkage:

chr18:0-24500000 → chr18:44800000-78077248

chr5:0-107400000 → chr18:44800000-40400000 (4.40M) → chr5:107400000-180915260

chr16:0-75500000 → chr18:24500000-40400000 (15.90M) → chr16:75500000-90354753

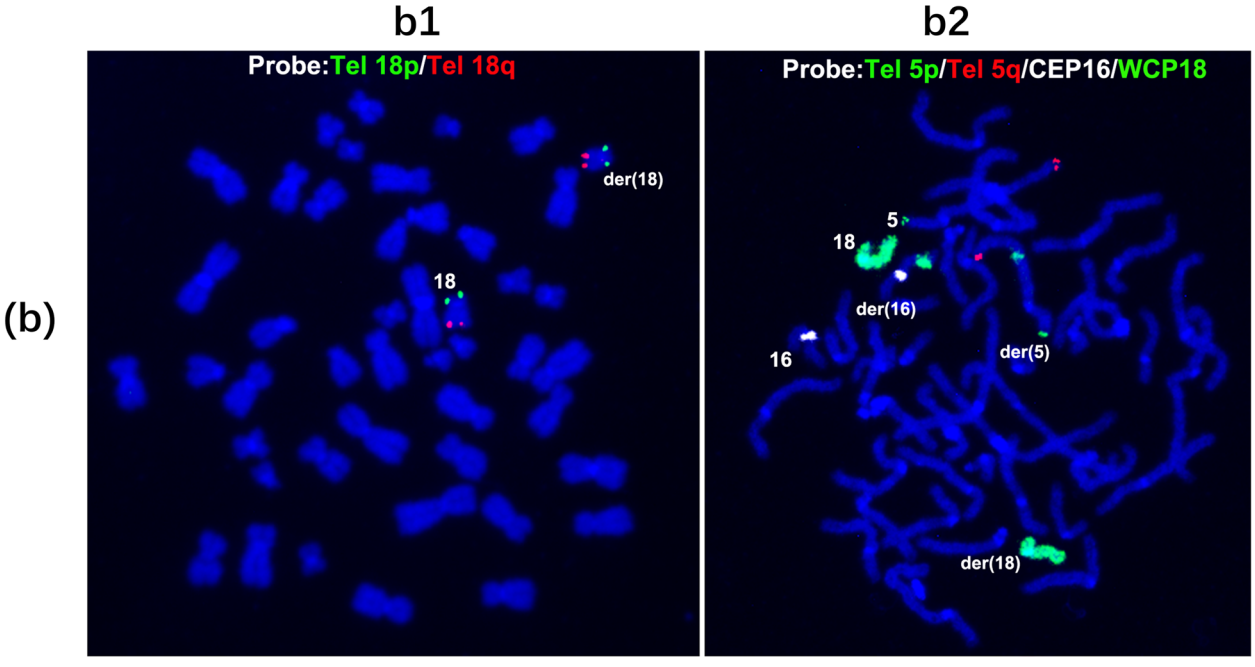


Fig. 5 (See legend on next page.)

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Fig. 5 The karyotype and breakpoint of the female chromosome insertion were corrected by C-Moka and verified by FISH. **A** Reassembly of all chromosomal linkages and breakpoint positions that were involved in the insertions according to C-Moka images of the female. **B** FISH images of the female. B1: FISH using the Tel 18p(green)/Tel 18q(red) probe combination with female peripheral blood mid-metaphase phases showed the following results: A total of 20 mid-metaphase phases were observed, and 2 normal signals were observed for each probe in each metaphase phase. B2: FISH using the probe combinations Tel 5p(green)/Tel 5q(red)/CEP 16(white)/WCP 18(green) with the female peripheral blood metaphase phase separately showed the following results: A total of 20 metaphase phases were observed, and one der(5), one der(16) and one der(18) chromosome were observed in each plate

amniotic fluid and tumor. So, it can be used as an effective means to analyze chromosomal rearrangements.

Although the odds of having a balanced or normal embryo are low, PGT-SR reduces the risk of miscarriage and is the only way to help CCRs carriers give birth to related offspring. Moreover, in the present study, we observed that only 15% (3/20) of the CCRs female had balanced embryos. Therefore, it was necessary to use comprehensive chromosome analysis to perform genetic testing. With C-Moka, reads from translocated chromosomes can be captured directly and normal reads can be captured on non-translocated chromosomes. Haplotypes can be constructed without the need for lineage and reference embryo pre-laboratory experiments, and can be used to distinguish chromosomal anomalies in MaReCs, simplifying the process and saving costs. In this study, without the involvement of non-maternal families or reference embryos, multiple breakpoints on three insertional chromosomes in female CCRs were directly identified using C-Moka analysis. Reads on the insertional chromosomes were captured, and normal reads were captured on non-insertional chromosomes, so as to successfully construct the haplotype in one step. This simplifies the PGT-SR process and saves patient costs.

Although C-Moka technology shows its high resolution and accuracy, it still has its limitations. C-Moka is applicable to translocations and insertions above 1 Mb, inversions above 5 Mb, and genomic CNVs above 100 Kb, and is not applicable to heterozygotes and variants in highly repetitive regions of the genome, such as

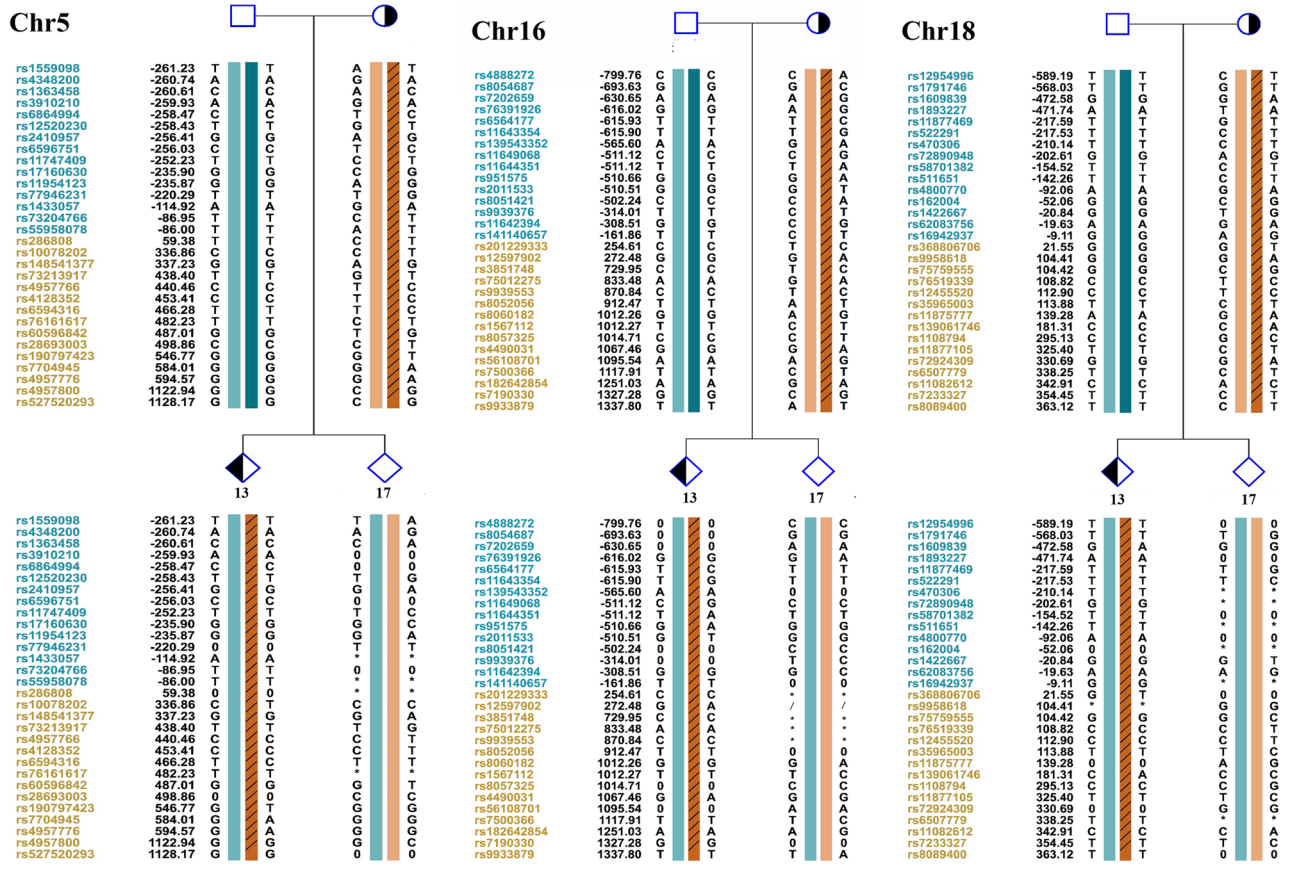

Fig. 6 SNP-based haplotyping. The reference SNP cluster ID numbers are listed on the left side. The dark cyan and mustard of ID numbers refer to the downstream and upstream informative SNPs of the chromosomal location of the breakpoint, respectively. The left slash is the SNP haplotype linked to the breakpoint of the female chromosome. The results show that E13 is the carrier of the complex chromosome translocation that matches the female karyotype and E17 is a karyotypically normal embryo

Table 2 Comprehensive comparison of technical and practical features among conventional cytogenetic, Low-Pass CNV-seq, CMA and C-Moka

	Conventional cytogenetic	Low-pass CNV-seq (0.5X)	CMA	C-Moka
Balanced translocation	3-5 Mb	×	×	1 Mb
Robertsonian translocation	✓	×	×	✓
Inversion	3-5 Mb	×	×	5 Mb
Insertion	5 Mb	×	×	1 Mb
CCRs	5 Mb	×	×	1 Mb
Copy number variation (CNV)	5 Mb	100 kb	50 kb	100 kb
Level of mosaicism	5%	20%	/	20%
Breakpoint resolution of structural variants (SV)	5 Mb	×	×	100 kb
Breakpoint resolution of copy number variations (CNV)	×	150 bp	1 kb	20 kb
Regions of homozygosity (ROH) / Uniparental disomy (UPD)	×	10 Mb	10 Mb	5 Mb
Dynamic mutation	×	×	×	×
Instrument configuration	incubator、chromosome harvester、chromosome slide preparer and scanning system	high-throughput sequencer、bioinformatics server、nucleic acid extractor、PCR instrument	Capture chip, Hybridization system、Chip scanning system	high-throughput sequencer、bioinformatics server、nucleic acid extractor、PCR instrument
Sample requirements	High (requires viable cells)	Low	Low	Medium (requires intact cells)
Sample processing time	15–25 days	15 days	15 days	25 days
Cost	40 to 120 USD	200 to 350 USD	400 to 550 USD	450 to 550 USD

heterochromatin regions of the centromeres, telomere, satellite DNA regions, etc. The applicable scenarios, advantages, and disadvantages of the four genetic testing technologies—conventional cytogenetics, Low-Pass CNV-seq, CMA and C-Moka—are summarized in Table 2. Based on both literature and our clinical case findings, C-Moka is recommended in specific scenarios: when conventional karyotyping detects ambiguous or seemingly balanced rearrangements suggestive of complex abnormalities; upon observation of recurrent segmental aneuploidies affecting identical chromosomal regions across multiple PGT-SR embryos; and in cases where familial genetic testing or PGT points to previously unrecognized structural rearrangements, particularly when breakpoint confirmation or haplotype construction is essential. It should be noted, however, that the adoption of C-Moka may be limited by its substantial cost, with testing fees typically ranging from $450 to $550 USD per assay. In contrast, conventional cytogenetic methods remain more suitable for large-scale population screening due to their significantly lower cost.

This case highlights a critical diagnostic oversight: the recurrent findings of deletions and duplications at 18q11.2, 18q12.3, and 18q21.1 across multiple embryos—including Embryos 1, 2, and 4 in the first PGT-SR cycle and Embryos 7, 8, 10, 11, and 12 in the second—did not initially raise clinical suspicion of possible multi-breakpoint rearrangements on chromosome 18 in a parental carrier. It was not until the third PGT-SR cycle, during which additional embryos with aberrations in these same regions were identified, that a CCR was suspected and later confirmed. This likely explains the markedly low euploidy rate observed in the embryos from this patient. These findings underscore the importance for clinicians to consider the possibility of a parental chromosomal rearrangement involving multiple breakpoints on a single chromosome—and the value of the C-Moka technique—when repeated segmental aneuploidies affecting the same chromosomal regions are detected across multiple embryos to avoid diagnostic delays and improve embryo selection strategies.

Conclusion

Accurate chromosomal karyotype identification and breakpoints mapping are the keys to provide prediction for fertility risk, genetic counseling, and fertility guidance for couples who carry CCRs. In the study, a cost-effective and highly accurate technique, the C-Moka technique, was successfully applied to assist in the diagnosis of CCRs and directly identify the breakpoints and construct haplotypes to successfully identify carrier embryos from normal embryos in the absence of family lineage and reference embryo pre-tests. Our study illustrates the C-Moka technique combining with PGT-SR is a valuable tool in assisted reproduction for couples with complex chromosomal abnormalities and recurrent miscarriages.

Abbreviations

C-Moka	The Chromosome conformation based Karyotyping technique
CCRs	Complex chromosomal rearrangements
PGT-SR	Preimplantation genetic testing for chromosomal structural rearrangements
SVs	Structural variants
FISH	Fluorescence in situ hybridization
WGS	Whole genome sequencing
MaReCs	Mapping allele with resolved carrier status
CMA	Chromosome microarray analysis
CNV-Seq	Copy number variation sequencing
SNP	Single nucleotide polymorphism
NGS	Second-generation sequencing
IVF	In vitro fertilizations
ICSI	Intracytoplasmic sperm injection
Mb	Megabase

Supplementary Information

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Supplementary Material 1.

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Authors' contributions

Tingting Zheng: Writing – original draft, Funding acquisition，Investigation, Methodology, Visualization，Conceptualization. Dehua Cheng: Methodology, Resources. Yuxia Yang: Methodology, Resources. Sijia Lu: Methodology, Resources. Huiying Li: Investigation. Liangqun Xie: Visualization, Formal analysis. Qinhua Li: Conceptualization. Hong Ye: Project administration, Funding acquisition, Supervision.

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Data availability

The high-throughput sequencing results of 20 biopsies of human early blastocyst trophoblast cells analyzed during this study are publicly available in the National Center for Biotechnology Information BioSample database under accession number PRJNA128600 (https://www.ncbi.nlm.nih.gov/sra/PRJNA1286004). The raw sequence reads are accessible via the SRA under submission ID SUB15415567.

Declarations

Ethics approval and consent to participate

This study complied with the Declaration of Helsinki and was approved by the Reproductive Medicine Ethics Committee of Yichang Central People's Hospital (Approval No. 2024-305-01, September 25, 2024). All participants were over the age of 18. Written informed consent was obtained from all participants to take part in this study and for this information to be published.

Consent for publication

Written informed consent was obtained from the patient for publication of this case report and accompanying images.

Competing interests

The authors declare no competing interests.

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