



Identification of cryptic balanced translocations in couples with unexplained recurrent pregnancy loss based upon embryonic PGT-A results

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Abstract

Purpose The goal of this study is to determine whether any balanced translocation (BT) had been missed by previous karyotyping in patients with unexplained recurrent pregnancy loss (uRPL).

Methods This case series included 48 uRPL-affected couples with normal karyotypes. The embryos from these couples have all undergone preimplantation testing for aneuploidies (PGT-A). Based on the PGT-A's results, 48 couples could be categorized into two groups: 17 couples whose multiple embryos were detected with similar structural variations (SVs, segmental/complete) and 31 couples without such findings but who did not develop any euploid embryo despite at least three high-quality blastocysts being tested. The peripheral blood sample of each partner was then collected for mate-pair sequencing (MPseq) to determine whether any of them were BT carriers.

Results MPseq analyses identified 13 BTs in the 17 couples whose multiple embryos had similar SVs detected (13/17, 76.47%) and three BTs in the 31 couples without euploid embryo obtained (3/31, 9.7%). Among the 16 MPseq-identified BTs, six were missed due to the limited resolution of G-banding karyotyping analysis, and the rest were mostly owing to the similar banding patterns and/or comparable sizes shared by the two segments exchanged.

Conclusion A normal karyotype does not eliminate the possibility of carrying BT for couples with uRPL. The use of PGT-A allows us to perceive the “carrier couples” missed by karyotyping analysis, providing an increased risk of finding cryptic BTs if similar SVs are always detected on two chromosomes among multiple embryos. Nonetheless, certain carriers with translocated segments of sub-resolution may still go unnoticed.

Keywords Cryptic balanced translocation · GTG-banded karyotype analysis · Mate-pair sequencing · Missed diagnosis · Preimplantation genetic testing for aneuploidies · Unexplained recurrent pregnancy loss

Introduction

Balanced translocation (BT) is defined as the exchange of two segments originating from two or more non-homologous chromosomes [1]. The prevalence is about 1.6 to 2 per 1000 newborns [2] but significantly increased to 3~7% in couples with recurrent pregnancy loss (RPL) [3]. BT carriers are prone to experience adverse pregnancies and spontaneous miscarriages due to the formation of quadrivalents and the resultant segregation modes that may produce unbalanced gametes. It has been reported that the proportion of normal

haploid sperms and oocytes produced by BT carriers is 45% [4] and 30% [5], respectively, suggesting that more than half of the gametes are abnormal. Therefore, knowing the carrier status thus enables the otherwise unwary couple to be informed of such risk and of the various reproductive options available to them.

Parental karyotyping analysis remains the first-tier clinical strategy for screening BT carriers [3]. However, its maximum resolution can only reach about 4 Mb (under ideal conditions) and it still has limited capability in detecting submicroscopic structural variations (SVs) [1]. As for the alternatives, fluorescence in situ hybridization (FISH) requires prior knowledge of target regions of chromosomes for probe design, whereas chromosomal microarray analysis (CMA) does not possess the ability to detect the

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balanced changes. Next-generation sequencing (NGS) has been recently used for the detection of balanced translocations and inversions; nevertheless, the mainstream of current NGS relies on the construction of small-insert DNA libraries (200~500 bp), which limits the detection capability of BTs, especially in those cases mediated by repetitive elements [6]. Despite the longer read-length (even up to 250 kb [7]) being a natural advantage for BT detection, third-generation sequencing technologies are not particularly well-suited for the purpose of carrier screening due to the cost factors. In comparison, mate-pair sequencing (MPseq), an optimized protocol of NGS, provides improved sensitivity in detecting balanced SVs by utilizing longer DNA fragments (from ~1 to 10 kb). Multiple laboratories have demonstrated its capability in detecting BTs with favorable results reported [8, 9]. Based on our previous work [10], MPseq provided a higher detection yield of balanced translocations and inversions (up to 11.7%) than the routine G-banded karyotyping analysis. However, whether it could be served as the first-tier test for RPL-affected populations (Table 1; [11–17]), remains inconclusive.

Cryptic balanced translocations [1] refer to those that are easily missed or not readily identified by conventional cytogenetic analysis, usually because of (i) (sub)telomere translocations, the segment(s) translocated is/are with a small size, near to or below the resolution limit (e.g., only a G-light band is visible in the translocated segment(s), thus rendering it difficult to recognize or being mistakenly reported as chromosomal polymorphism); (ii) two segments exchanged are characterized by comparable sizes and similar banding patterns; and (iii) complex chromosomal rearrangements, which involve three or more chromosomes or having more than two breakpoints. Once cryptic BT carriers with fertility intentions were missed, their RPLs are typically considered “unexplained” (uRPL). When the individuals in this subpopulation seek medical assistance, a potential strategy to prevent miscarriages might involve the use of in vitro fertilization (IVF) combined with preimplantation genetic testing (PGT) to rule out the embryos with aneuploidies (PGT-A). Their embryos, like those from carriers with apparently BTs, are expected to present with “unbalanced translocation-like” characteristics (similar SVs are always detected on two chromosomes among multiple embryos) from PGT-A analysis [18, 19]. In this regard, the “unbalanced translocation-like” embryos might be an indicator for further follow-up for these individuals. Additionally, the confirmation of carrier status in these patients might also be helpful to subject the embryos to PGT for structural rearrangements (PGT-SR) in their future IVF cycles. While it is noteworthy that the segments of cryptic BTs are often with small size and different clinics/laboratories adopt a wide range

of resolutions for PGT-A (ranging from 1 to 20 Mb), as well as the fact that there must be an adequate number of embryos to be aware of such characteristic, carriers of cryptic BT may still exist in couples in whom such embryonic characters were not observed.

In this study, 48 couples with normal karyotypes who underwent PGT-A for uRPL were included for MPseq.

Patient profile

This case series consisted of 48 couples who were diagnosed with RPL (defined as the occurrence of three or more clinical pregnancy losses) (Table 2). They were gradually found and noticed by us during the clinical consultation process from November 2017 to December 2020 owing to their shared dilemma of facing the tough decision-making process of selecting the most suitable embryo for transfer with PGT-A results available.

Previous RPL workup

As recommended by the American Society for Reproductive Medicine (ASRM) [3], we firstly administered an RPL workup for them.

G-banded karyotyping analysis

Cell culture, smear preparation, and Giemsa staining were performed according to standard procedures [20]. At least five cells from two separate cultures were analyzed, and at least two images from each karyotype were saved. The initial reviews were completed independently by two qualified cytogeneticists and confirmed by the laboratory chief. Karyotypes were described following the ISCN guideline (2016) [21]. Results were reported at a resolution of 350~550 bands [22].

Other tests in RPL workup

The other tests contained in the RPL workup included the assessments of female sex hormones, prolactin, and thyroid-related hormones (via chemiluminescence assay, Roche-Diagnostics, Basel, Switzerland), alongside HbA1c (via liquid chromatography, Bio-Rad, Hercules, CA, USA), thrombophilia markers, and antiphospholipid antibodies (via coagulation tests, chromogenic assays, and turbidimetry, Diagnostica Stago, Asnières sur Seine, France). The endometrial conditions (e.g., endometritis) were determined based on hysteroscopy and immunohistochemical analyses of specimens obtained from endometrial biopsy and curettage.

Table 1 Four mainstream technologies for detecting balanced chromosomal rearrangements

Technologies	Introduced	Delineation of breakpoint-junction in single nucleotide level?	Technical specification	Current price per case (USD)
Karyotype-banded	1970s, T Caspersson and L Zech et al. [11], ME Drets and MW Shaw et al. [12]	No, ~5–10 Mb	Classic cytogenetics; allow the visualization for all 23 chromosome pairs; incapable of detecting certain types of aberrations	\$90–250
Fluorescence in situ hybridization (FISH)	1980s–1990s, ML Pardue and JG Gall et al. [13]	No, < 1 Mb to gene-level	Specific to one or a few loci; probe design requires prior knowledge of target chromosomal regions	\$300–550
Next-generation sequencing (NGS, e.g., mate-pair sequencing)	Late 2000s, E Kawashima, L Farinelli and P Mayer et al. [14]	Yes	Cost-effective; enable to detect of the > 5-Mb absence of heterozygosity; detection of rearrangements is limited whether they were mediated by highly repeated elements	0.4 × WGS (human genome), ~\$150; 30 × WGS (human gene), ~\$500 + [15]
Third-generation sequencing (TGS, also known as long-read sequencing)	2010s, J Korlach and SW Turner et al. [16]	Yes	Possess natural advantages for translocation detection; breakpoints can be pinpointed even if the rearrangements were mediated by short repeating elements (such as LINE); costly and with relatively high error rate	Prices vary widely, and costly when with the same read-depth from the NGS platform
Optical genome mapping (OGM)	1990s, Dr. David C. Schwartz and the team [17]	Only with assistance of gap-PCR and Sanger sequencing	Non-sequencing method (comparison of the labeling pattern in case from the reference control); longest DNA size (> 150 kb) for analysis allows identification of rearrangements mediated by low-copy repeat	> \$1000

Table 2 Baseline characteristics of the couples in the two categories

	No. of couples	17
Category-1	Maternal age (years)	32.18 ± 2.31
	Maternal BMI (kg/m ²)	23.81 ± 2.93
	Paternal age (years)	32.47 ± 3.24
	Paternal BMI (kg/m ²)	26.21 ± 5.04
	No. of previous miscarriages	4.43 ± 1.28
	No. of couples	31
Category-2	Maternal age (years)	31.77 ± 4.31
	Maternal BMI (kg/m ²)	23.07 ± 3.66
	Paternal age (years)	31.90 ± 4.62
	Paternal BMI (kg/m ²)	27.24 ± 3.74
	No. of previous miscarriages	3.97 ± 0.83

BMI body mass index

After the completion of the above examinations, the couples had the following features in common: (i) maternal age was under 38 years old; (ii) received negative findings from the G-banded karyotype analysis; (iii) unexplained causes of RPL despite a comprehensive workup as proposed by the ASRM. In light of the uRPL being a common indication for PGT, the PGT-A procedures were subsequently added into their IVF cycles.

NGS-based PGT-A and clinical findings prior to the enrollment

NGS-based PGT-A

The protocols of controlled ovarian hyperstimulation (COH) and oocyte retrieval had been described in our previous study [23]. After being fertilized via intracytoplasmic sperm injection (ICSI), zygotes were cultured in vitro to the blastocyst stage (5–6 days). Several cells of trophectoderm (TE) were harvested from the blastocysts and the rest of the embryonic components were cryopreserved until a decision was made based on PGT-A results. All couples in this study had at least three high-quality (> 4BC, Gardner's standard [24]) blastocysts being tested. Their TE samples were washed three times in polyvidone and transferred to the EP tubes containing 2.5 µL phosphate buffer solution for subsequent DNA extraction and single-cell whole genome amplification (WGA, SurePlex DNA Amplification System, Illumina, San Diego, CA, USA). Sequencing proceeded on Illumina MiSeq platform (VeriSeq PGS Kit, MiSeq Reagent Kit, Illumina, San Diego, CA, USA). For all full or partial chromosomal regions detected, the difference value (DV) parameter was generated by calculating “control normalized mean coverage for one copy” multiplied by “expected ploidy,” plus “sample normalized mean coverage.” The embryos with DVs

below 20% and above 80% were defined as euploidy and aneuploidy, and those with DVs in-between were classified as mosaic embryos. Deletions and duplications of chromosomal segments larger than 4 Mb were reported.

Clinical findings

Upon receiving PGT-A results, the embryos of a total of 17 couples were observed with abovementioned “unbalanced translocation-like” characteristics (hereafter referred to as “category-1”). The results for all embryos from the 17 couples are listed in Supplementary Table 1. As cryptic BTs often involve small translocated segments (< 5 Mb), we proposed MPseq prior to their embryo transfer to determine whether they were BT carriers and to avoid the risks of cryptic imbalances in their presumably balanced or euploid embryos at the current PGT-A's resolution of 4 Mb.

Despite the embryos of the remaining 31 couples (hereafter referred to as “category-2”) did not exhibit apparent “unbalanced translocation-like” characteristics, none of them developed into euploid embryos. As the female partners in these couples were with young age and had obtained at least three high-quality blastocysts being tested, we found it is difficult to give them a medically grounded explanation. Due to the fact that these couples can only select mosaic embryos for transfer in addition to initiating a new COH cycle, we also recommended MPseq for them for seeking any additional information to potentially guide their next steps.

Diagnostic interventions

The study was approved by the Ethics Committee of the Reproductive Medicine Center of Shandong University ([2017] IRB No. 121), and all couples provided written informed consent before the peripheral blood collection.

Mate-pair sequencing

Genomic DNA (gDNA) from each partner was extracted with the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and quantified with the Qubit dsDNA HS Assay kit (Invitrogen, Carlsbad, CA, USA), and the DNA integrity was evaluated by agarose gel electrophoresis. After QC, 1 µg DNA (measured by Qubit) from each sample was sheared with an E220e Focused-Ultrasonicator with miniTUBE Red (Covaris, Woburn, MA, USA) into 3–8 kb (median size of 5 kb) fragments and subjected to mate-pair library construction according to our previous study [25]. At least 60 million read-pairs were generated for each sample, equivalent to an average of 4× read-depth on the MGISEQ-2000 platform (MGI Tech Co., Ltd., Shenzhen, China). Thereafter, copy number variations (CNVs at 50-kb),

SVs (10-kb), and regions with absence of heterozygosity (AOHs, 5-Mb) were identified through our reported bioinformatics pipelines [26], and they were further annotated and interpreted based on the guidelines of the American College of Medical Genetics and Genomics (ACMG).

Verification of the translocations

Breakpoint junction-specific PCR with Sanger sequencing was used to verify the translocations detected by MPseq (all primer sequences designed for this study are listed in Supplementary Table 2), while FISH was employed if there were commercial probes (the Vysis locus-specific identifier DNA probe, Abbott Molecular Inc., Des Plaines, IL, USA) available.

Characterization of translocations detected in two categories

In total, MPseq identified 16 BTs in 16 couples, all of which were validated by breakpoint-junction specific PCR with Sanger sequencing and/or FISH. Thirteen BTs were revealed in 13 couples from category-1 (13/17, 76.47%), and three BTs in three couples from category-2 (3/31, 9.68%) (Table 3).

In category-2, the translocations identified in 20SD0026 and 20SD0082 both involved a cryptic segment that was smaller than 4Mb the PGT-A's resolution, while both segments of the translocation identified in 20SD0002 were smaller than 4 Mb. It might explain why the previous PGT-A did not indicate any apparent “unbalanced translocation-like” characteristics from the embryos (Fig. 1). Previous PGT-A results for the three couples were provided in Supplementary Table 3.

In category-1, the translocations identified in samples 20SD0016, 20SD0040, and 20SD0093 were missed mainly because one or both segments were with a size near to or smaller than the detection limit of karyotyping analysis (6~9 Mb). The translocations found in samples 20SD0001, 20SD0004, 20SD0007, 20SD0030, and 20SD0080 were missed possibly because the two segments shared a similar banding pattern and/or comparable size. In the case of samples 20SD0023, 20SD0024, 20SD0029, and 20SD0091, only a barely visible G-dark band could be observed in one of the translocated segments because of unsatisfactory terminal staining, whereas the other segment contained only a G-light band, which may elucidate why the previous karyotyping analysts failed to report them. The last translocation in sample 20SD0076 was likely missed due to the limited banding level and poor staining quality in the chromosome preparation. No complex chromosomal rearrangement was found in this study. Of note, the involvement of bands 4p16, 8q24.2, and 13q33 were reported twice in different samples,

and the segments involving these bands were with a size almost near to the limit of detection, or located adjacent to the telomeric regions (Fig. 1).

Recognition of chromosome regions with AOHs

Based on our newly developed bioinformatic algorithm [26], MPseq also revealed the regions with AOHs that involved known imprinted genes and exceeded 5 Mb in size (Supplementary Table 4). There were multiple AOH regions found in four samples, while the overall sizes observed in three samples (20SD0001, 20SD0029, and 20SD0077) did not meet the reported cut-off for parental consanguinity, typically around ~89 Mb. In comparison, a total of eight AOH regions were identified in sample 20SD0042 with a combined size of 154.4 Mb, suggesting that the parents of this male patient probably married consanguineously. Given that the presence of AOHs potentially contributes to miscarriages, further mechanism studies are warranted.

Follow-up of pregnancy outcomes

We also conducted follow-up studies on the 16 couples with cryptic BTs identified (Cat.1 = 13; Cat.2 = 3).

As indicated by MPseq, the existing PGT-A platform's resolution was feasible for the segments carried by the 13 couples in category-1, whose earlier PGT-A results could be adopted directly. Ten of them were with euploid embryos transferred. As current resolution (4 Mb) was insufficient, three couples in category-2 chose to initiate their second cycle but pursued PGT-SR. Based on the exact locations and flanking sequence contexts of knowing the breakpoint junctions from MPseq, their embryos with aneuploidies, balanced karyotypes, and normal karyotypes were distinguished as previously described [27]. Results showed that only the couple 20SD0026&27 had euploid embryo (balanced) obtained and transferred.

Among the 11 couples who underwent embryo transfers, a total of 11 clinical pregnancies (11/11, 100%) were achieved with an outcome of two miscarriages (2/11, 18.18%) and nine live births (9/11, 81.82%). The live birth rate (LBR) of “carrier couples” in this study was higher (81.82%) than the average LBR reported (59.28% [28]) for euploidy transfers in younger women.

Timeline

A timeline for the entire diagnostic and treatment process of these couples is depicted in Supplementary Fig. 1.

Table 3 An overview of 16 cryptic balanced translocations identified by mate-pair sequencing

	Sample ID	Karyotypes	Molecular karyotypes reported by MPseq	Sizes of translocated segments		Suspected reasons for missed detection by karyotyping analysis	Pregnancy outcomes
				ChrA (Mb)	ChrB (Mb)		
Category-1	SD-RPL_20SD0001	46,XX	46,XX,t(8;18) (p23.1;p11.21)	11.45	12.22	Similar banding patterns	A healthy female infant with a balanced karyotype was born
	SD-RPL_20SD0004	46,XX	46,XX,t(6;10) (q23.3;p12.1)	33.16	25.67	Similar banding patterns	A healthy female infant with a balanced karyotype was born
	SD-RPL_20SD0007	46,XX	46,XX,t(7;9) (q32.3;q31.1)	27.82	35.46	Similar banding patterns	A healthy female infant with a balanced karyotype was born
	SD-RPL_20SD0016	46,XY	46,XY,t(4;10) (p16.1 ^a ;q26.2)	7.16	6.09	Near to the lower limit	Embryo has not been transferred yet
	SD-RPL_20SD0023	46,XY	46,XY,t(8;11) (q24.22 ^b ;p15.4)	11.83	7.31	One was stained too lightly at the end, while the other involved only a G-light band	A healthy male infant with a balanced karyotype was born
	SD-RPL_20SD0024	46,XX	46,XX,t(2;8) (p24.1;q24.21 ^b)	19.51	18.79	One was stained too lightly at the end, while the other involved only a G-light band	Only untransferable embryos with 'unbalanced translocation-like' characters were obtained
	SD-RPL_20SD0029	46,XY	46,XY,t(1;19) (p36.22;p13.2)	9.81	12.61	One was stained too lightly at the end, while the other involved only a G-light band	A healthy female infant with a balanced karyotype was born
	SD-RPL_20SD0030	46,XX	46,XX,t(11;13) (q24.3;q32.3 ^c)	7.09	14.12	Similar banding patterns	A healthy male infant with a balanced karyotype was born
	SD-RPL_20SD0040	46,XY	46,XY,t(4;5) (p15.3;p15.33)	11.30	4.50	Below the lower limit	A healthy female infant with a balanced karyotype was born
	SD-RPL_20SD0076	46,XY	46,XY,t(4;8)(p12;q21.3)	46.58	56.61	Limited banding level	Clinical miscarriage
	SD-RPL_20SD0080	46,XY	46,XY,t(1;13) (q42.2;q33.2 ^c)	15.32	8.94	Similar banding patterns	Embryo had been transferred and the patient is now pregnant
	SD-RPL_20SD0091	46,XX	46,XX,t(7;14) (q34;q32.12)	16.04	12.65	One was stained too lightly at the end, while the other involved only a G-light band	A healthy male infant with a balanced karyotype was born
	SD-RPL_20SD0093	46,XX	46,XX,t(7;16) (p22.1;q23.2)	5.65	9.18	Near to the lower limit	Clinical miscarriage
Category-2	SD-RPL_20SD0002	46,XX	46,XX,t(2;4) (p25.3;p16.3 ^a)	0.31	3.73	Below the lower limit	Failed to obtain transferable embryo
	SD-RPL_20SD0026	46,XX	46,XX,t(1;4) (p36.23;q35.2)	7.91	2.16	Below the lower limit	A healthy female infant with a balanced karyotype was born
	SD-RPL_20SD0082	46,XX	46,XX,t(3;7) (p25.3;q36.3)	10.01	0.99	Below the lower limit	Failed to obtain transferable embryo

a ~ c: The translocated segments that occurred twice in different samples

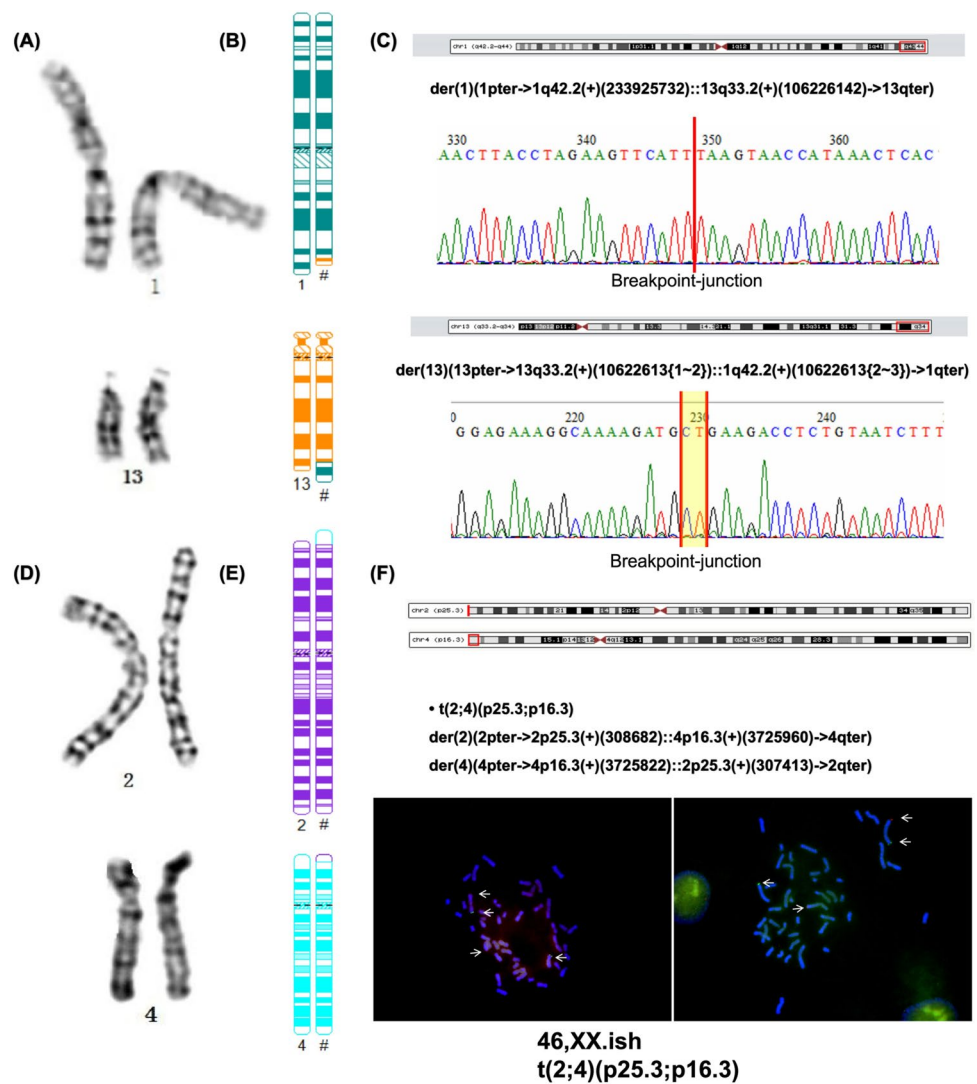
Chr chromosome

Review of literature on (sub)telomeric/cryptic rearrangements associated with RPL

Based on the results retrieved, the publications regarding cases

with (sub)telomeric/cryptic rearrangements were more commonly found in diseases like intellectual disabilities (ID) [29] or congenital anomalies [30, 31], etc., while the studies focusing solely on RPL were relatively limited. Most of these studies are the reports that consist of a few to several dozen

Fig. 1 Two cryptic BTs identified by MPseq. **A** to **C** show the detection result in sample 20SD0080 (from category-1), while **D** to **F** indicate the result in sample 20SD0002 (from category-2). **A**, **D** Previous G-banded chromosomes; **B**, **E** ideograms of normal and derivative chromosomes; **C**, **F** translocated segments indicated in UCSC browser. Red line indicates the breakpoint junction, while 2-bp microhomology is highlighted in yellow. In figure **F**, white arrows indicate the fluorescent signals in normal and derivative chromosomes. BT, balanced translocation; MPseq, mate-pair sequencing; FISH, fluorescence in situ hybridization



cases, which were predominantly published around the early 2000s, a time when FISH technique was clinically mature and widely promoted. This might be due to the fact that the individuals studied in the former category were typically genetically unbalanced and phenotypically noticeable, leading to a well-defined molecular diagnosis. In comparison, couples affected by RPL tend to be genetically balanced, with no evident phenotypic manifestations other than fertility problems, thus resulting in the difficulty of diagnosis (Table 4; Supplementary Fig. 2).

In these studies, parental carriers of cryptic BT were identified by tracing their abnormal fetuses or offspring with birth defects. In a study led by Shaffer et al. [33], a couple with a history of RPL and loss of an infant because multiple abnormalities underwent FISH testing; thus, the father was identified as a carrier of cryptic BT involving chromosomes 7 and 11. Brackley et al. [35] reported a case where the father was detected to carry a cryptic BT between the long arms of chromosomes 2 and 7 in relation to multiple

fetal anomalies observed in his wife's pregnancies. Similarly, five cryptic BT carriers were found in five families with a history of RPL and/or offspring with unbalanced karyotypes by Wakui et al. [34]. Bacino et al. [36] reported on a 4 ½-year-old girl with severe mental retardation and minor anomalies, who inherited the unbalanced product of a cryptic translocation involving chromosomes 2 and 17 from her father. According to a study from Kilby et al. [37], the prenatal FISH test of the chorionic villous sampling cultures showed the presence of an unbalanced karyotype, 46, XX,ish der(7) t(2;7)(q37;q36)pat derived from the balanced subtelomeric translocation in the father. Joyce et al. [41] described two families, whose offspring were affected by Miller-Dieker syndrome (MDS). The molecular analysis of two mothers revealed cryptic BTs between the subtelomeres of 11p and 17p. Bruyere et al. [43] reported a young woman who had experienced three spontaneous miscarriages and two neonatal deaths. The woman was demonstrated as the carrier of cryptic BT between the long

Table 4 Summary of literature on (sub)telomeric/cryptic rearrangements associated with recurrent pregnancy loss

Study	Country/region	Year of publication	Has previous karyotyping been performed?	Type of banding technique and resolution of the image	Method of verification	Number of couples/subjects	Number of positive cases/carriers	Detection rate	Translocated segments and the estimated sizes	Number of couples who had borne children with birth defects	Related diseases
Horacio Rivera, et al. [32]	England	1995	Yes (positive)	G-banding; Not mentioned	FISH	16	0	0%	–	Unclear	Unclear
Lisa G. Shaffer, et al. [33]	America	1996	Yes (negative)	G-banding; Not mentioned	FISH	1	1	100%	7q35 (11.24 Mb), 11q23.3 (13.81 Mb)	1	Multiple congenital anomalies; Recurrent miscarriages
Keiko Wakui, et al. [34]	Japan	1999	Yes (positive/negative)	G-banding; Not mentioned	FISH	5	2	40%	5p15.1 (15 Mb), 10p13 (12.2 Mb); 7q36.2 (4.04 Mb), 13q34 (4.87 Mb)	4	Multiple congenital anomalies; Holoprosencephaly; Cat cry syndrome; Recurrent miscarriages
Karen J. Brackley, et al. [35]	England	1999	Yes (negative)	G-banding; Not mentioned	FISH	1	1	100%	2q36 (12.2 Mb), 7q37 (2.1 ~2.3 Mb)	1	Consecutive congenital fetal anomalies; Recurrent miscarriages
Carlos A. Bacino, et al. [36]	America	2000	Yes (negative)	G-banding; Not mentioned	FISH	1	1	100%	2qter (?), 17q23 (18.6 Mb)	1	Congenital anomalies; mental retardations; Recurrent miscarriages; Stillbirths
M. D. Kilby, et al. [37]	England	2001	Yes (negative)	G-banding; Not mentioned	FISH	1	1	100%	2q37 (12.2 Mb), 7q36 (6.54 Mb)	1	Consecutive congenital fetal anomalies
Brigitte Benzacken, et al. [38]	France	2002	Yes (negative)	G/R-banding; Not mentioned	FISH	50	0	0%	–	Unclear	Recurrent miscarriages
Yao-Shan Fan, et al. [39]	England & Canada	2002	Yes (negative)	G-banding; 450 ~ 500 bands	FISH	80	0	0%	–	Unclear	Recurrent miscarriages
Sezin Yakut, et al. [40]	Turkey	2002	Yes (negative)	G-banding; Not mentioned	FISH	5	1	20%	3qter (?), 10pter (?)	1	Recurrent miscarriages

Table 4 (continued)

Study	Country/region	Year of publication	Has previous karyotyping been performed?	Type of banding technique and resolution of the image	Method of verification	Number of couples/subjects	Number of positive carriers	Detection rate	Translocated segments and the estimated sizes	Number of couples who had borne children with birth defects	Related diseases
Christine A. Joyce, et al. [41]	England	2002	Yes (negative)	G-banding; 500–550 bands	FISH	2	2	100%	11p15.5 (2.8 Mb), 17p13.3 (3.3 Mb)	2	Miller-Dieker syndrome; Recurrent mis-carriages
Annette E. Cockwell, et al. [42]	England	2003	Yes (negative)	G-banding; 550 bands	FISH	50	4	8%	13q1?1 (?), 15q11.2 (76.83 Mb); 3q?29 (?), 10p?15.3 (?); 3q?29 (?), 10p?15.3 (?); 21pter (?), Ypter (?)	Unclear	Recurrent mis-carriages
Helene Bruyere, et al. [43]	Canada	2003	Yes (negative)	G-banding; Not mentioned	array-CGH	1	1	100%	2q37.2 (5.9 Mb), 17q25 (7.53 Mb)	1	Neonatal deaths; Recurrent mis-carriages
Syed M Jalal, et al. [44]	America	2003	Yes (negative)	G-banding; 550 bands or higher	FISH	53	0	0%	–	Unclear	Recurrent mis-carriages
Fowzan S. Alkuraya, et al. [45]	Greece	2006	Yes (negative)	G-banding; Not mentioned	FISH	1	1	100%	10qter (?), 17qter (?)	1	Multiple congenital anomalies; Recurrent mis-carriages
Sandra Monfort, et al. [46]	Spain	2006	Yes (negative)	G-banding; 400–500 bands	FISH	18	1	5.56%	2pter (?), 3pter (?)	0	Recurrent mis-carriages
Jasen L. Wise, et al. [47]	America & Canada	2009	Yes (negative)	G-banding; ≥ 500 bands	FISH	565	0	0%	–	0	Phenotypically normal
Burak Durmaz, et al. [48]	Turkey	2013	Yes (negative)	G-banding; Not mentioned	FISH	32	0	0%	–	0	Recurrent mis-carriages

Table 4 (continued)

Study	Country/region	Year of publication	Has previous karyotyping been performed?	Type of banding technique and resolution of the image	Method of verification	Number of couples/subjects	Number of positive cases/carriers	Detection rate	Translocated segments and the estimated sizes	Number of couples who had borne children with birth defects	Related diseases
Amani Hajlaoui, et al. [49]	Tunisia	2018	Yes (negative)	R-banding; Not mentioned	FISH	21	1	4.76%	3q28 (5.72 Mb), 4p16 (6 Mb)	13	Congenital abnormalities; idiopathic intellectual disabilities; recurrent miscarriages
Zirui Dong, et al. [10]	China	2019	Yes (positive/negative)	G-banding; 320–550 bands	MPseq	1077	8/19	0.74%/1.76%	–	Unclear	Recurrent miscarriages
Shuo Zhang, et al. [50]	China	2023	Yes (negative)	G-banding; Not mentioned	OGM	11	11	100%	–	Unclear	Abnormal pregnancy history; Recurrent miscarriages

FISH fluorescence in situ hybridization, *array-CGH* array-comparative genomic hybridization, *MPseq* mate-pair sequencing, *OGM* optical genome mapping

arms of chromosomes 2 and 17. In a study by Alkuray et al. [45], a 36-year-old Greek woman with a history of three early miscarriages gave birth to a male infant with multiple anomalies in her last pregnancy. She was diagnosed with a cryptic BT between 10q26.3 and 17q25. In another study by Hajlaoui et al. [49], FISH was employed for 21 clinically normal couples exhibiting a “normal” karyotype with at least two abortions and detected one cryptic rearrangement between chromosomes 3q and 4p in the female partner of a couple.

There are several other studies that investigated couples only affected by RPL. Yakut et al. [40] performed FISH analysis on five families who had suffered at least five prior miscarriages. One couple was found to carry a cryptic BT between chromosomes 3 and 10, while in another couple, a signal of chromosome 20 was observed in the D-group chromosomes. Cockwell et al. [42] conducted a re-evaluation (FISH) of 50 couples with normal karyotypes who experienced three or more miscarriages. One female patient was identified to carry a subtelomeric BT, and five patients (one female and four males) were found to have cryptic abnormalities involving the pericentromeric regions of acrocentric chromosome. In a study by Monfort et al. [46], 18 couples with normal karyotypes who had suffered four or more spontaneous miscarriages were subjected to FISH analysis. A cryptic BT between 2 and 3p was detected in a woman with a history of seven miscarriages. Cryptic BTs were not reported in these studies [32, 38, 39, 44, 47, 48].

With the introduction of second- [10] and third-generation sequencing [50] since the 2010s, an increasing number of studies are taking advantage of these new technologies for the validation of cryptic BTs.

Discussion

Our findings demonstrated the significance of PGT-A for the subpopulation of couples with undetermined causes of RPL due to the cryptic BT in one of the partners. Once “carrier couples” were missed in the initial screening round of parental karyotyping, the use of PGT-A can be taken as an opportunity to re-identify them.

BT is presently one of the few well-established etiologies that is strongly associated with RPL; however, some of them could not be readily diagnosed by the gold-standard test (karyotyping analysis) [51]. PGT-SR has been demonstrated to be a management option for effectively reducing miscarriage rates in carrier populations [52]. Given the limitations of chromosome banding techniques, observation of embryonic patterns may provide reassurance against the possibility of missing cryptic BT carriers if the couples decide to pursue pregnancy through IVF and PGT. According to a previous study, Sundheimer et al. [18] identified BTs in eight couples

with exactly the same mutations as indicated by their “unbalanced translocation-like” embryos, while most of the subjects they included were not subjected to parental karyotyping prior to IVF, which limits our knowledge of the spectrum of the cryptic BTs missed by G-banded chromosome analysis.

Despite the embryonic patterns of PGT-A could be regarded as an indicator of parental carrier status, there were still three cryptic BT carriers identified in category-2. Two were with one translocated segment cryptic to the PGT-A’s resolution, while the other one was with both segments that were smaller than the cutoff. The inability to obtain transferable euploid embryo despite multiple high-quality blastocysts being tested may also present a potential indication for finding cryptic BTs. In a recent study [50], a total of 11 cryptic BTs were uncovered, with seven out of the 22 translocated segments being smaller than the routine resolution of PGT-A (5 Mb). Although a small size of the unbalanced segment may less likely to result in a lethal consequence (embryonic demise), adverse birth outcomes are often reported. A segmental aneuploidy of larger than 2% of haploid autosomal length (HAL) is highly lethal and often leads to intrauterine death, while an unbalanced segment less than 1% of the HAL may not be lethal but could increase the risks of birth defects [53]. A substantial amount of evidence has been demonstrated that cryptic imbalances/rearrangements are responsible for multiple congenital anomalies, such as mental retardation [31, 54], leukemia [55], autism spectrum disorder [56], and fetal ultrasound abnormalities [57]. In these studies, karyotyping analysis had been performed on a large proportion of subjects in advance, with most of them having “normal” karyotypes. The resolution of PGT-A in our study was enhanced to 4 Mb, which is higher than that adopted in other studies (e.g., 5–10 Mb [58]), while there is still a fraction of translocated segments measured between 1 and 5 Mb [59]. If one (out of four) segment exchanged is with a size below the detection range or there were limited embryos obtainable for testing, the embryonic patterns unique to BT carriers may also not be indicated by PGT-A, thus resulting in the adverse outcomes listed above.

Before the advent of NGS and third-generation sequencing technologies, the method most used for BT detection is FISH. We selected MPseq to identify cryptic BTs in this study since there was no prior knowledge of target chromosomes for probe selection in category-2 (PGT-A’s results did not point to the derivative chromosomes). Furthermore, as a test that can only pinpoint a few loci, FISH confers a risk of missing BT again if PGT-A-indicated chromosomes do not match the chromosomes that translocation truly involved. Additionally, as cryptic BTs often involve small translocated segments (<5 Mb, almost 1/3 in our study), the genomic contexts and structure of BT obtained from MPseq could also be served

as the basis for subsequent PGT-SR to discern embryos with small-segment rearrangements. Third-generation sequencing technologies [60, 61] can accomplish the same goal, but the related testing costs are considerably higher.

Limitations remain. In this study, a relatively higher detection rate of cryptic BTs (13/17 in category-1 and 3/31 in category-2) was observed in uRPL patients. However, it should be noted that this observation was based on a small sample of susceptible individuals and may not accurately represent the true prevalence of cryptic BTs in the whole population with uRPL. Given the fact that nearly all current research on cryptic rearrangements are in the form of the case report. We expect our study to contribute to future systematic reviews/meta-analyses in this field, thus allowing for an accumulated number of cryptic BT cases. In fairness, MPseq analysis is not perfect either. Specifically, it has limited capability of detecting Robertsonian translocations and the recurrent $t(11;22)(q23;q11.2)$ [62] due to its unsatisfactory performance in the extensive length of repetitive sequences, which has been known to be the limitation of currently available molecular technologies [63]. In some cases (e.g., with similar banding patterns), the identification of cryptic BT could also be achieved by the use of C-banding, silver nitrate (AgNOR) staining, or by improving the resolution of the initial method. In clinical settings, the product of conception (POC) of some couples with uRPL could be preserved sometimes. “Unbalanced translocation-like” POCs might be another indicator of the existence of cryptic BT. Nevertheless, to recognize the “unbalanced translocation-like” characteristics, a minimum accumulation of two to approximately three POCs is required, which would result in a longer turnaround time (more than one PGT cycle). In addition, the patients need to be educated and well-informed to make them aware of the importance of preserving POCs; the potential impact of maternal cell contamination should also be considered. In contrast, the use of PGT-A allows us to view the genetic results of multiple embryos in a shorter time, enabling it easier to realize this characteristics. For those “carrier couples” missed by karyotyping analysis, the stress and anxiety of failing to determine the cause of RPL, as well as the additional expenses for consultations, examinations, and transportation, merit attention too. Regrettably, we neglected to collect questionnaires from them, which is another limit of our study.

Conclusion

To conclude, clinicians taking care of patients with uRPL should be aware that a normal karyotype does not eliminate the possibility of a BT. In the setting of the presence of embryos with “unbalanced translocation-like” characteristics

following PGT-A, there is a potential for MPseq to provide valuable information with a significant impact on the reproductive choices of couples seeking medical attention for RPL.

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Author contribution S. L., H. L., Z.-J. C., K. W. C., Z. D., and J. Y. designed the whole study. J. Y. recruited patients. S. L. collected samples and variant verification and data curation. H. Li contributed to FISH validation. Z. D. and K. W. C. completed MPseq, data analysis, and variant interpretation. Y. G., Y. Z., and X. Y. collected and analyzed the PGT results. S. L., H. L., K. W. C., Z. D., and J. Y. wrote the manuscript. All authors approved the final version of the manuscript.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

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