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GENETICS

Analysis of molecular cytogenetic features and PGT-SR for two infertile patients with small supernumerary marker chromosomes

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Abstract

Research question Can preimplantation genetic testing for structural rearrangement (PGT-SR) with next-generation sequencing (NGS) be used to infertile patients carrying small supernumerary marker chromosomes (sSMCs)?

Design In this study, two infertile patients carrying ring sSMCs were recruited. Different molecular cytogenetic techniques were performed to identify the features of the two sSMCs, followed by clinical PGT-SR cycles.

Results The results of G-banding and FISH showed that patient 1's sSMC originated from the 8p23-p10 region, with a resulting karyotype of [47,XY, del(8)(p23p10), +r(8)(p23p10).ish del(8)(CEP8+,subtle 8p+,subtle 8q+),r(8)(CEP8+,subtle 8p-,subtle 8q-)[55/60].arr(1-22) ×2,(X,Y)×1]. The sSMC of patient 2 was derived from chromosome 3 and further microdissection with next-generation sequencing (MicroSeq) revealed it contained the region of chromosome 3 between 93,504,855 and 103,839,892 bp (GRCh37), which involved 52 known genes. So the karyotype of patient 2 was 47,XX, +mar.ish der(3)(CEP3+,subtle 3p-,subtle 3q-)[49/60].arr[GRCh37] 3q11.2q13.1(93,500,001_103,839,892) ×3(0.5). PGT-SR with NGS was performed to provide reproductive guidance for the two patients. For patient 1, four balanced euploid embryos and four embryos with partial trisomy/monosomy of (8p23.1-8p11.21) were obtained, and a balanced euploid embryo was successfully implanted and had resulted in a healthy baby. For patient 2, an embryo with monosomy of sex chromosomes and another embryo with a duplication at (3q11-q13.1), neither of which was available for implantation.

Conclusions The identification of the origins and structural characteristics of rare sSMCs should rely on different molecular cytogenetic techniques. PGT-SR is an alternative fertility treatment for these patients carrying sSMCs. This study may provide directions for the assisted reproductive therapy for infertile patients with sSMC.

Keywords Small supernumerary marker chromosome (sSMC) · Chromosome microdissection · Next-generation sequencing · Preimplantation genetic testing

Introduction

Small supernumerary marker chromosomes (sSMC) refer to chromosomal fragments or markers that are structurally abnormal and difficult to identify by G-banding cytogenic techniques [13, 16]. sSMCs are typically smaller than chromosome 20 on a same metaphase and have been identified in ~0.072% of prenatal cases and ~0.125% of infertile couples [3, 12]. Crolla et al. reported that in 137 cases of sSMCs, 59% were mosaics and approximately 70% were identified as de novo. Most sSMCs are derived from acrocentric chromosomes, and approximately 40% of sSMCs are known to involve chromosome 15 [6, 10]. Patients carrying sSMCs that contain only the centromere or heterochromatin regions usually do not present with clinical manifestations.

sSMCs derived from a non-proximal centromere are rare and generally contain autosomal fragments without telomere structures. These sSMCs often present as ring chromosomes and can be passed down to offspring [11]. Patients carrying this type of sSMC have a higher risk of clinical phenotypes compared to sSMCs derived from a proximal centromere. The

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phenotypes of patients with sSMCs vary significantly from normal to extremely serious, based on the origin and size of the euchromatin fragment and the genes associated with the sSMC [14]. Some patients carrying sSMCs do not have obvious clinical features. However, the abnormal pairing and segregation of chromosomes during meiosis may produce unbalanced gametes with abnormal chromosome numbers or structures, which can lead to infertility, recurrent miscarriages, or infants with severe defects, including developmental delays, mental retardation, epilepsy, or complex deformities [4, 7, 9, 11, 18]. Additionally, offspring that inherit sSMCs from their parents may also have fertility problems [15]. Manvelyan et al. reported that approximately half of infertile patients carrying sSMCs inherited them from their parents [13]. In vitro fertilization (IVF)/PGT-SR is widely used for couples with fertility problems. Therefore, it is important to establish the origin of sSMCs in these parents. To the best of our knowledge, there are only a few reports on the use of PGT-SR for infertile couples carrying sSMCs.

In this study, G-banding, FISH and MicroSeq techniques were used to identify the origin and structural characteristics of sSMCs from two infertile patients. In each case, PGT-SR was performed, and in one case, a healthy embryo was successfully implanted.

Materials and methods

Patients

Two infertile patients carrying sSMC that visited our clinic were recruited in this study, in 2015 and 2016, respectively.

Patient 1 was a 25-year-old male who had been married for two years. His partner had failed to conceive, despite unprotected sexual intercourse with ejaculation. This patient had been born at full term with a normal Apgar score into a nonconsanguineous family. At the time of this study, he exhibited a normal appearance, intelligence, and body type, and was 165 cm tall and weighed 55 kg. He had normal secondary sexual characteristics and his external genital organs were well-developed. Routine semen analysis of patient 1 revealed severe oligozoospermia. Reproduction-related examinations revealed normal hormone levels and no azoospermia factor (AZF) microdeletions on the Y chromosome.

Patient 2 was a 29-year-old female who was 158 cm tall and weighed 69 kg. She failed to become pregnant after four years of marriage with a normal sex life and no contraception. She began to menstruate regularly at 13 with intervals of approximately 30 days. Physical examination revealed normal mental development, mammary glands, and female external genitalia. The results of multiple hormone tests, including follicle-stimulating hormone, luteinizing hormone, testosterone, and estradiol, were normal. Salpingography indicated an obstruction of one of the fallopian tubes.

This study was conducted with the informed consent of the two couples and was approved by the Ethics Committee of the Reproductive and Genetic Hospital of Citic-Xiangya.

Karyotyping and FISH analysis

Peripheral blood from the two patients was collected, and lymphocytes were cultured for G-banding and FISH analyses. Metaphase spreads were analyzed by GTG-banding using trypsin and Giemsa. Karyotype analyses were performed using standard methods according to the International System for Human Cytogenetic Nomenclature (ISCN 2016) [23]. FISH analyses used commercially available subtelomeric-specific probes for patient 1 (Subtel 8p (green)/Subtel 8q (red)/CEP 8 (indigo); pseudo color is white) and CEP 3 (Red) and subtelomeric probes[Subtel 3p (green)/Subtel 3q (red)]of chromosome 3 for patient 2, which were purchased from Abbott Co. (Abbott-Vysis, USA). The FISH procedures were carried out according to the manufacturer's instructions and are described in our previous report [5].

Single nucleotide polymorphism microarray analysis

Genomic DNA was extracted from the patients' peripheral blood using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The DNA concentrations were measured using a NanoDrop spectrophotometer (ND-1000, Thermo Fisher Scientific, USA). Experiments were performed using Cytoscan 750 K chips (Affymetrix, Santa Clara, CA, USA), according to the manufacturer's instructions. The results were scanned and analyzed using the Chromosome Analysis Suite (CHAS, Affymetrix).

MicroSeq of marker chromosomes

The FISH results for patient 2's sSMC did not show any signal for the telomere region. To further determine its structure, MicroSeq of the ring sSMC was performed. The basic experimental steps were performed as we have described previously [8] with the following modifications: chromosome microdissection was carried out in a water droplet (which enables the transfer of 100% of the dissected target chromosome fragments to the collection solution in Eppendorf tubes) and the traditional DOP-PCR amplification method was replaced with a WGA4 kit (Sigma-Aldrich, USA). Before microdissection, 10 μl of deionized water was added to the G-banding metaphase chromosome sample area. Eight copies of the labelled chromosomes were removed from the water droplet and transferred to 9 μl of collection solution (deionized water) and then amplified according to the instructions provided with the

WGA4 kit. The quality of the amplified product was evaluated by 2% agarose gel electrophoresis.

The sequencing library for the amplified product was generated according to the instructions of the Ion Xpress Library Kit (Life Technologies, USA). The library was diluted to 2.5 pg/μl and amplified using the Ion PGM Template OT2 200 Kit for 5.5 h. The Ion Personal Genome Machine (PGM) system (Ion PGM Sequencing 200 Kit v2) was employed for sequencing. The universal forward and reverse primer sequences (CCGACTCGAG) were removed from the sequencing results and the sequences were then mapped to the human genome (GRCh37) using the Burrows-Wheeler Aligner (BWA). A breakpoint was defined as a location in which the number of sequences that aligned to that region was significantly higher than all of the other regions.

Preimplantation genetic testing of aneuploidy with CCS

PGT-SR-CCS was carried out for the two couples. The procedures, including promoting ovulation, ovum collection, insemination, embryo culture, blastocyst biopsy, and embryo cryopreservation, have been reported previously [8]. Five to eight trophectoderm cells were obtained by laser-assisted blastocyst biopsy. Whole genome amplification of the embryo biopsy samples was performed using a single-cell PicoPLEX WGA kit (Rubicon Genomics) and the products were analyzed by next-generation sequencing (NGS) for whole genome copy numbers to identify balanced or unbalanced embryos. Each DNA library was then sequenced on a Life Tech. Ion Proton system with 318 chips as paired-end 200-bp reads. Image analysis and base calling was performed using a Life Tech. 460 Flow system.

Results

The G-banding results showed that both patients 1 and 2 carried mosaic karyotypes with the presence of sSMCs (Figs. 1a and 2a). The FISH results showed that patient 1's sSMC originated from the 8p23-p10 region, with a resulting karyotype of [47,XY, del(8)(p23p10), +r(8)(p23p10).ish del(8)(CEP8+,subtle 8p+,subtle 8q+),r(8)(CEP8+,subtle 8p-,subtle 8q-)[55/60].arr(1-22) ×2,(X,Y)×1] (Fig. 1a–c). Further SNP microarray analysis showed that patient 1 displayed normal copy numbers (Fig. 1d). Eight embryos were obtained from patient 1 and his partner for PGT-SR, including four embryos with normal copy numbers, two embryos with partial trisomy (8p23.1-8p11.21), and two embryos with partial monosomy (8p23.1-8p11.21) (Fig. 1d). An embryo with a balanced karyotype was selected and successfully implanted. Prenatal examination by amniocentesis at 18 weeks of pregnancy showed that the chromosome karyotype of the fetus was normal. A healthy male infant was born at full term and the child was healthy upon follow-up at 2.5 years of age.

The results of FISH and MicroSeq revealed that patient 2's sSMC originated from chromosome 3 and contained the 93,504,855–103,839,892 bp region of chromosome 3 (GRCh37) (Fig. 2b–d). This region includes 52 known genes, seven of which have been recorded in the OMIM database as leading to phenotypes if mutations are present (Fig. 2e). Follow-up SNP analysis showed that there was a mosaic duplication of approximately 50% of this region (Fig. 2f), which combined with the results of G-banding and FISH indicated that the karyotype of the patient was47,XX, +mar.ish der(3)(CEP3+,subtle 3p-,subtle 3q-)[49/60].arr[GRCh37] 3q11.2q13.1(93,500,001_103,839,892) ×3(0.5). Two embryos were obtained from patient 2 and her partner, including one with monosomy of sex chromosomes and one with a duplication of the (3q11-q13.1) region (Fig. 2g) by PGT-SR-CCS. Based on these results, no normal copy number embryo was available for implantation.

Discussion

We report two infertile couples that came to our hospital for genetic and reproductive counselling, of whom one partner carried an sSMC. Different molecular cytogenetic techniques were used to identify the origin and the structural characteristics of the sSMCs in the two cases, and PGT-SR was performed for each couple.

The common type of sSMC(8) is derived from a non-proximal region of chromosome 8. sSMC(8) carriers with clinical phenotypes mainly display mosaic or recessive mosaic karyotypes. It has been reported that almost all sSMC(8)s with determined structural characteristics contain part of the short arm, part of the long arm and the complete centromere region of chromosome 8 (http://ssmc-tl.com/chromosome-8.html, Accessed 2 Feb 2019). By contrast, we report an sSMC(8) that lacks a telomere, is derived from a normal chromosome 8, and contains only part of the short arm and a partial centromere, with the remainder of chromosome 8 reconnected to form a derivative chromosome (Fig. 1a). To the best of our knowledge, this type of karyotype with sSMC(8) (47,XY, del(8)(p23p10), +r(8)(p23p10).ish del(8)(CEP8+,subtle 8p+,subtle 8q+),r(8)(CEP8+,subtle 8p-,subtle 8q-)[55/60].arr(1-22) × 2,(X,Y) × 1) has not been reported. Clinical phenotypes in individuals carrying sSMC(8) are caused mainly by unbalanced karyotypes. Patients with sSMC(8) originating from a proximal 8p manifest clinical phenotypes such as developmental delay, mental retardation, dysmorphic face, and autism, while those originating from an acrocentric 8q- mainly manifest as dysmorphic face, developmental delay and finger, or toe/foot malformations. In the present study, although the sSMC derived from chromosome

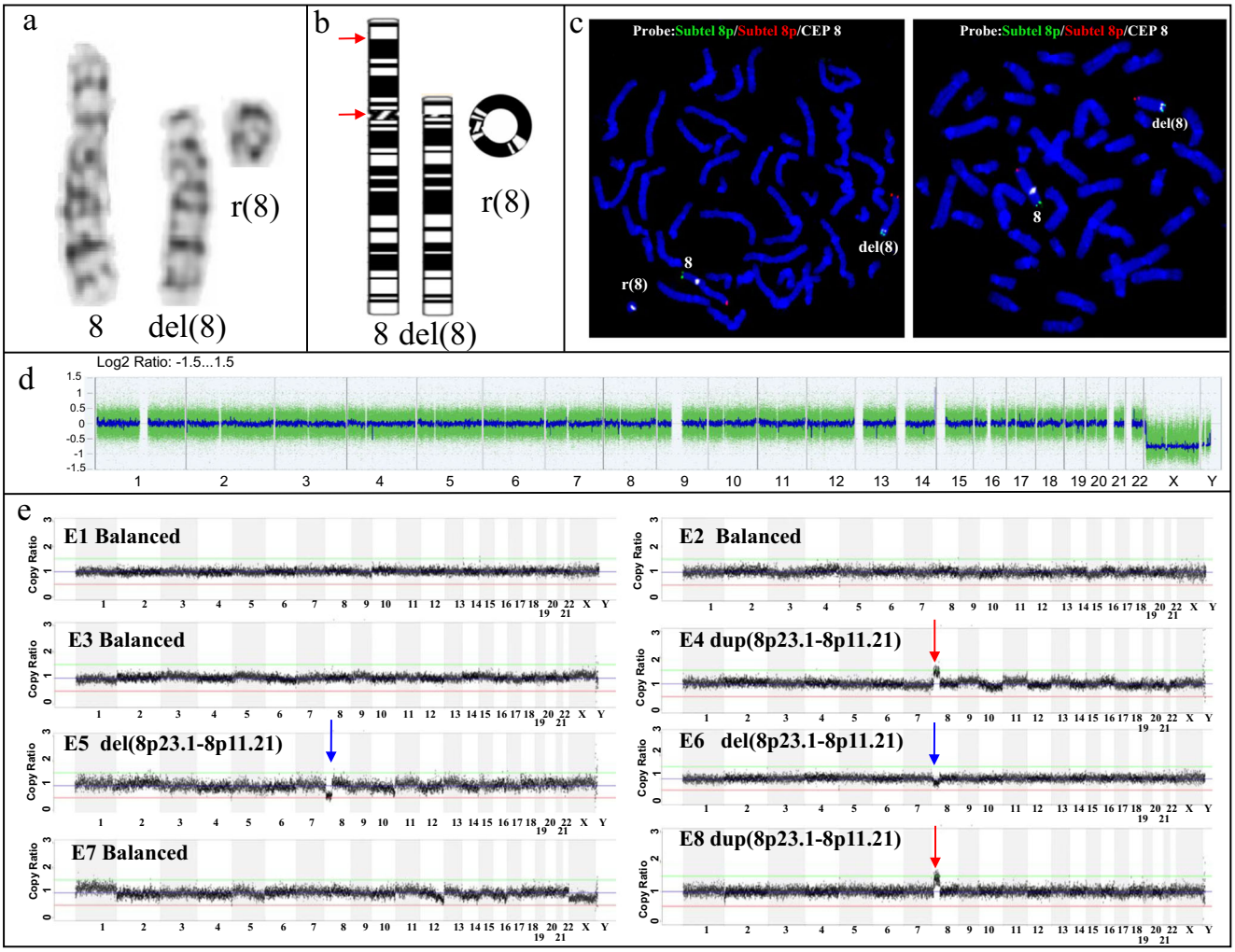

Fig. 1 sSMC characteristics and PGT-SR results for patient 1. **a**, **b** Partial karyotypes and ideograms of chromosome 8 and the observed del(8)/r(8). **c** FISH analysis with probes for chromosome 8, indicating the absence of the subtelomeric region in the r(8) and the presence of the subtelomeric region in the interstitial deletion del(8); chromosome 8 centromere-specific (red), short arm subtelomeric (green), and long arm subtelomeric (red). **d** SNP array results for genomic DNA from patient 1. **e** PGT-SR results for embryos E1-E8

8 (p23p10) should have originated from a proximal 8p, it was formed via the deletion of the 8p23-p10 region of a normal chromosome 8 and a complimentary ring. Furthermore, the abnormal karyotype was mainly 47,XY, del(8)(p23p10), +r(8)(p23p10), which accounted for 91.67% (55/60) of the metaphase chromosomes analyzed, whereas the karyotype without sSMC(8) accounted for only 8.33% (5/60). Moreover, the SNP analysis revealed that the genomic DNA copy number in the peripheral blood of patient 1 was normal (Fig. 1d) and had a balanced karyotype. These results may explain why patient 1 had no other obvious phenotypes.

The prevalence of sSMC(3) is lower and only fifty-four cases have been reported to date (http://ssmc-tl.com/chromosome-3.html, Accessed 2 Feb 2019). Of these, only 10 individuals carrying sSMC(3) had clinical phenotypes. In almost all of these cases, the individuals had developmental retardation, while a few others had serious deformities [20–22]. In our study, FISH analysis revealed that the sSMC(3) contained the centromere from chromosome 3 but did not include the telomere. To further clarify the molecular cytogenetic features, MicroSeq was used to isolate the sSMC, followed by amplification and sequencing. The sequencing data was aligned to the human genome and the results showed that patient 2's sSMC was a euchromatin fragment containing the 93,504,855-103,839,892 bp region of chromosome 3 (GRCh37). This region contains 52 known genes (Fig. 2e), in which the genes *PROS1, MINA, CPOX, DCBLD2, NFKBIZ, TFG,* and *TRMT10C* have been recorded in the OMIM database as having reported point mutations that can lead to disease. In addition, SNP microarray analysis showed that there was a mosaic duplication of approximately 50% of this region (Fig. 2f), which was consistent with the karyotype analysis. Previous study has defined the region of a centromere with no dose-sensitive genes as the critical non-

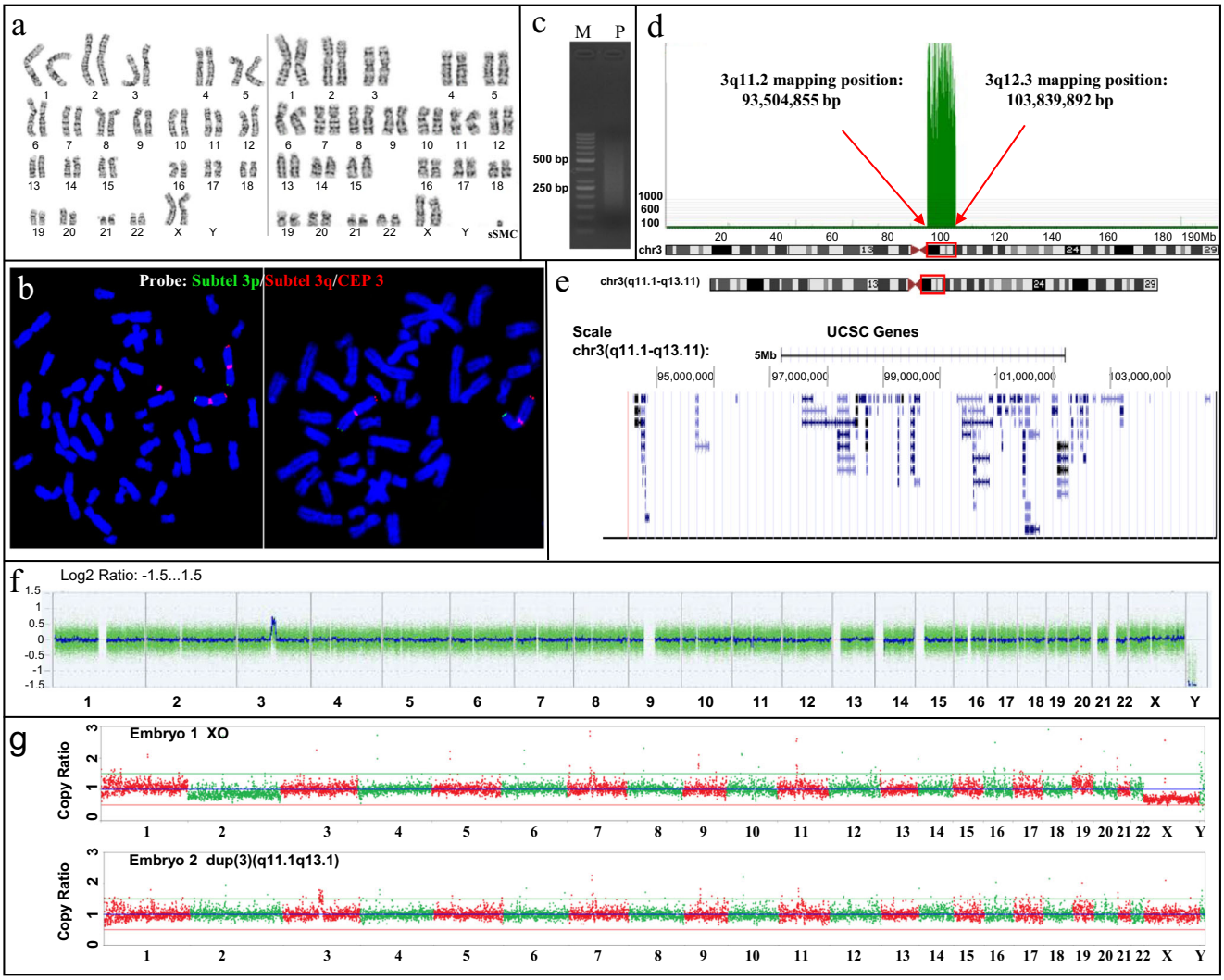

Fig. 2 sSMC(3) structural features and PGT-SR results for patient 2. **a** G-banding showed that the patient had a mosaic karyotype consisting of a normal karyotype (left) with an sSMC karyotype (right). **b** The sSMC chromosome showed a positive signal with the chromosome 3 centromere probe (CEP 3), without subtelomere 3p (Subtel 3p) and 3q (Subtel 3q) signal, indicating that it was derived from chromosome 3. **c** Microdissection of patient 2's sSMC(3) and gel electrophoresis analysis of the amplified products. **d** The NGS data of the sSMC compared to the chromosome 3 reference sequence revealed it was derived from the chr3:93,504,855-103,839,892 (GRCh37). **e** List of 52 known genes in the chr3:93,504,855-103,839,892 bp region. **f** SNP microarray results for genomic DNA from patient 2. The results showed that there was a mosaic duplication of 50% of the sSMC(3) fragment. **g** Embryo 1 was monosomy of sex chromosomes and embryo 2 had a partial duplication of the 3q11.1-3q13.1 fragment of chromosome 3

phenotypic region [1]. It has been reported that the range of the critical non-phenotypic region of the centromere of chromosome 3 is 73.17-105.48 Mb (http://ssmc-tl.com/chromosome-3.html, Accessed 2 Feb 2019). The sSMC(3) from patient 2 is located within the range of the critical non-phenotypic region. Thus, it is likely that none of genes in this region have dose-sensitive effects and that no known pathogenic genes are interrupted at the distal breakpoint of the long arm of this sSMC(3). These results may explain why patient 2 displayed no other obvious phenotypes.

PGT-SR is a reliable and effective IVF method for couples carrying abnormal chromosomes to have a child with a normal karyotype. Since the 1990s, FISH, array comparative genome hybridization (aCGH) and NGS have been used in PGT-SR to detect chromosomal copy numbers [2, 17, 19]. More recently, microdissection combined with NGS has been used to distinguish embryos with normal karyotypes from those carrying balanced translocations [8]. While PGT-SR-CCS is currently used to screen normal copy number embryos for couples with reciprocal translocations or for elderly couples, it is rarely used for couples carrying sSMCs.

Patients with ring sSMCs may produce gametes carrying different abnormal karyotypes due to different segregation patterns during meiosis (Fig. 3). When the combination of a ring chromosome and the corresponding chromosome with an interstitial deletion is passed on to the offspring, a balanced

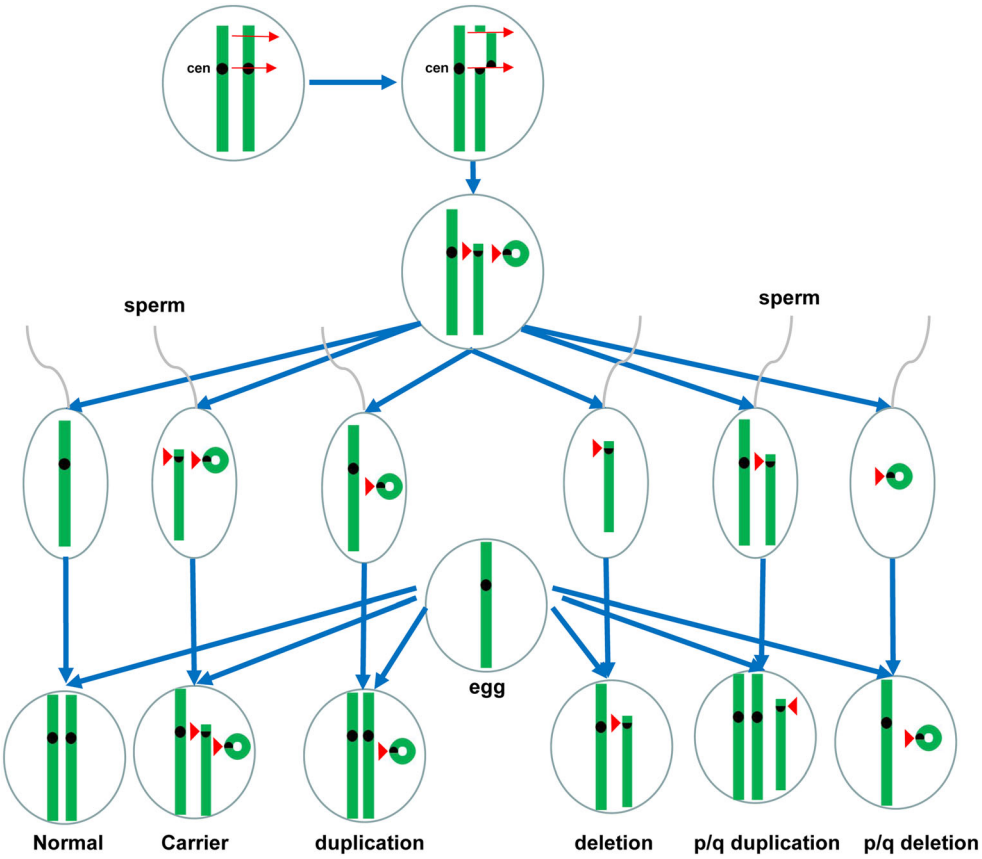

Fig. 3 Diagram of the separation pattern of ring-shaped sSMCs during meiosis

karyotype consisting of a complementary deletion and a ring chromosome is formed. When either a normal chromosome and the ring chromosome or only the chromosome with an interstitial deletion are transmitted to the gamete, which is then combined with another normal gamete from the partner, an unbalanced karyotype involving the corresponding partial trisomy or monosomy can be formed. These two types of abnormal chromosomes were detected in the blastocysts of our two patients, suggesting that there was a high risk that children with unbalanced karyotypes would be born if these couples had become pregnant naturally. In our study, NGS following a biopsy of trophectoderm cells from the blastocysts was used to detect the copy number of the embryos. Four normal copy number embryos were obtained from patient 1 and his wife. However, we could not distinguish embryos with normal and balanced karyotype by PGT-SR-CCS. Therefore, one of these embryos with highest rating scores successfully implanted. Furthermore prenatal diagnoses revealed that the fetus carried a normal karyotype, and follow-up examinations confirmed that the fetus developed normally after birth. By contrast, the two embryos obtained from patient 2 and her partner had unbalanced chromosomes (Fig. 2g). One embryo carried a monosomy of sex chromosomes, which will develop into a child with Turner's syndrome, and the other embryo carried a non-mosaic duplication of (3q11-q13.1), suggesting that this embryo inherited the sSMC(3) from the mother. It is difficult to predict the embryonic outcome and postnatal growth and development of this embryo, including whether the embryo would have no phenotype or if it would have a clinical phenotype. Therefore, with their full informed consent, this couple decided not to transplant either embryo and instead to continue the cryopreservation of the sSMC(3) embryo. The PGT-SR strategy based on the biopsy of the trophectoderm cells of the blastocysts combined with NGS successfully avoided the risk of implanting embryos with unbalanced karyotypes in these two couples, suggesting that this is an effective and reliable method for couples carrying sSMCs and that it can be applied in the future to other couples who are sSMC carriers.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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