



# Novel PGD strategy based on single sperm linkage analysis for carriers of single gene pathogenic variant and chromosome reciprocal translocation

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Received: 20 January 2020 / Accepted: 17 March 2020 / Published online: 29 April 2020  
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## Abstract

**Purpose** Preimplantation genetic diagnosis (PGD) analysis can be challenging for couples who carry more than one genetic condition. In this study, we describe a new PGD strategy to select which embryo(s) to transfer for two clinically challenging cases. Both cases lack essential family members for linkage analysis including de novo mutation combined with reciprocal translocation.

**Methods** Diverging from conventional method, we performed direct point mutation detection, quantitative analysis of gene copy number, combined with linkage analysis assisted by SNP information from single sperm (or polar bodies), thus establishing an all-in-one protocol for single embryonic cell preimplantation diagnosis for two co-existing genetic conditions (monogenic disease and chromosomal abnormality) on the NGS-based platform.

**Results** Using this newly developed method, 15 embryos from two cases were screened, and two embryos were determined as free of the monogenic disease and specific chromosomal abnormalities created by the prospective father's reciprocal translocations.

**Conclusion** This novel PGD strategy could effectively select unaffected embryo(s) for couples affected with or carrying a monogenetic disease and a reciprocal chromosome translocation concurrently.

**Keywords** PGD · Monogenic disorders · Reciprocal translocations · NGS · Sperm haplotyping

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**Electronic supplementary material** The online version of this article (<https://doi.org/10.1007/s10815-020-01753-2>) contains supplementary material, which is available to authorized users.

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## Introduction

So far, there are 6000–8000 known monogenic diseases in the human population. Many of them are severe congenital defects, and can even lead to premature death. Furthermore, the majority of them have no effective therapeutic options [1, 2]. Reciprocal translocations arise when two non-homologous chromosomes exchange their terminal segments and are the most common form of chromosomal abnormality, occurring in virtually 1/625 to 1/500 live births [26, 32]. The vast majority of these translocation carriers are phenotypically normal, because the majority of these types of rearrangements do not result in any significant deletion or duplication of chromosomal material [26, 34]. However, high rates of formation of unbalanced gametes can cause infertility, recurrent spontaneous abortions, and may lead to affected offspring [7, 23].

For challenging cases with monogenetic diseases and reciprocal translocations running in the same family, a prenatal diagnosis with amniocytes or chorionic villi is traditionally

used to directly examine the presence of monogenetic disease allele(s) with Sanger sequencing or dosage analysis by MLPA/quantitative PCR. Additionally, an examination of unbalanced translocation with cytogenetic methods like karyotyping, FISH, or CGH are conventionally recommended [11, 17]. However, due to the high risk of being pregnant with either or both genetic conditions, preimplantation genetic diagnosis (PGD) together with preimplantation genetic screening can plausibly improve pregnancy outcomes in these complicated cases. Traditional PGD detection methods include fluorescence in situ hybridization (FISH), polymerase chain reaction (PCR), multiplex quantitative PCR (qPCR), array-comparative genomic hybridization (aCGH), single nucleotide polymorphism (SNP) microarray, and karyotyping [7, 9, 14, 21, 28]. Recently, next generation screening (NGS) has emerged as a powerful tool applied in PGS or PGD [14, 17]. Each of the above methods, however, has its limitations and disadvantages on accuracy of diagnosis [7, 14, 17, 19].

Among the patients in our center, we encountered two families complicated with neurofibromatosis type II (NF2) and spinal muscular atrophy (SMA), respectively, coupled with reciprocal chromosome translocations in the prospective father. Neurofibromatosis (NF) is a relatively frequent autosomal dominant neurological disease across different ethnic groups. It includes two distinct types (Type I and Type II, respectively) [15]. NF type II (NF2) is caused by mutations of the *NF2* gene located on chromosome 22q12.2 (prevalence of ~1/100,000) [10, 33]. Spinal muscular atrophy (SMA) is an autosomal recessive childhood onset neuromuscular disease (prevalence of ~1/10,000, carrier frequency of ~1/50) [13, 21, 22]. Two highly homologous survival motor neuronal (SMN) genes, *SMN1*, and *SMN2*, are located in the 5q13 region and only differ in three intronic and two exonic nucleotides [5, 20, 29]. Only *SMN1* pathogenic variants can cause SMA [4, 22]. In 90–98% of SMA patients, homozygous deletion of exon 7 of the *SMN1* gene can be detected (even affecting exon 8) [22, 24, 29].

In this study, a novel approach was applied in two representative PGD cases. Besides monogenic diseases, both prospective fathers in these two families carry a specific reciprocal translocation. A common feature in these two cases is that the translocation found in both families started in the fathers (de novo). Therefore, haplotype information for paternal linkage analysis of the embryos cannot be derived. To solve this challenge, single sperm haplotyping is applied in this study. CNVs and SNVs information were obtained simultaneously from one embryo sample in a single sequencing run [11, 17, 31]. Combining information from single sperm haplotyping, NGS-based gene copy number quantitative analysis/pathogenic variants detection, SNP linkage analyses and chromosome aneuploidy analyses [25, 31, 32], we are able to select the appropriate embryos to transfer.

## Materials and methods

### Status of genetic conditions in the two study families

In case 1, the husband is affected with NF type II, positive for the *NF2* gene mutation (*NF2*, c.1574 + 2 T > C). At the same time, the husband is a carrier of a reciprocal translocation between Chr13 and Chr18 [t (13; 18) (q32; q21.3)], due to which the family has had three abnormal pregnancies. Skull hypoplasia and fetal edema appeared in the fetus at 13 weeks of the first pregnancy in 2013, and 14 weeks of the second pregnancy in 2014. The latest abnormal pregnancy occurred in 2016 and resulted in embryo damage at 11 weeks. In case 2, both partners are healthy carriers of the *SMN1* exons 7 and 8 deletion, and had one baby who was diagnosed with SMA and died at age 6 months. The husband has also been tested as a reciprocal translocation carrier [t (5; 14) (p15; q32)]. The pedigree charts of these two families are shown in Fig. 1a and b.

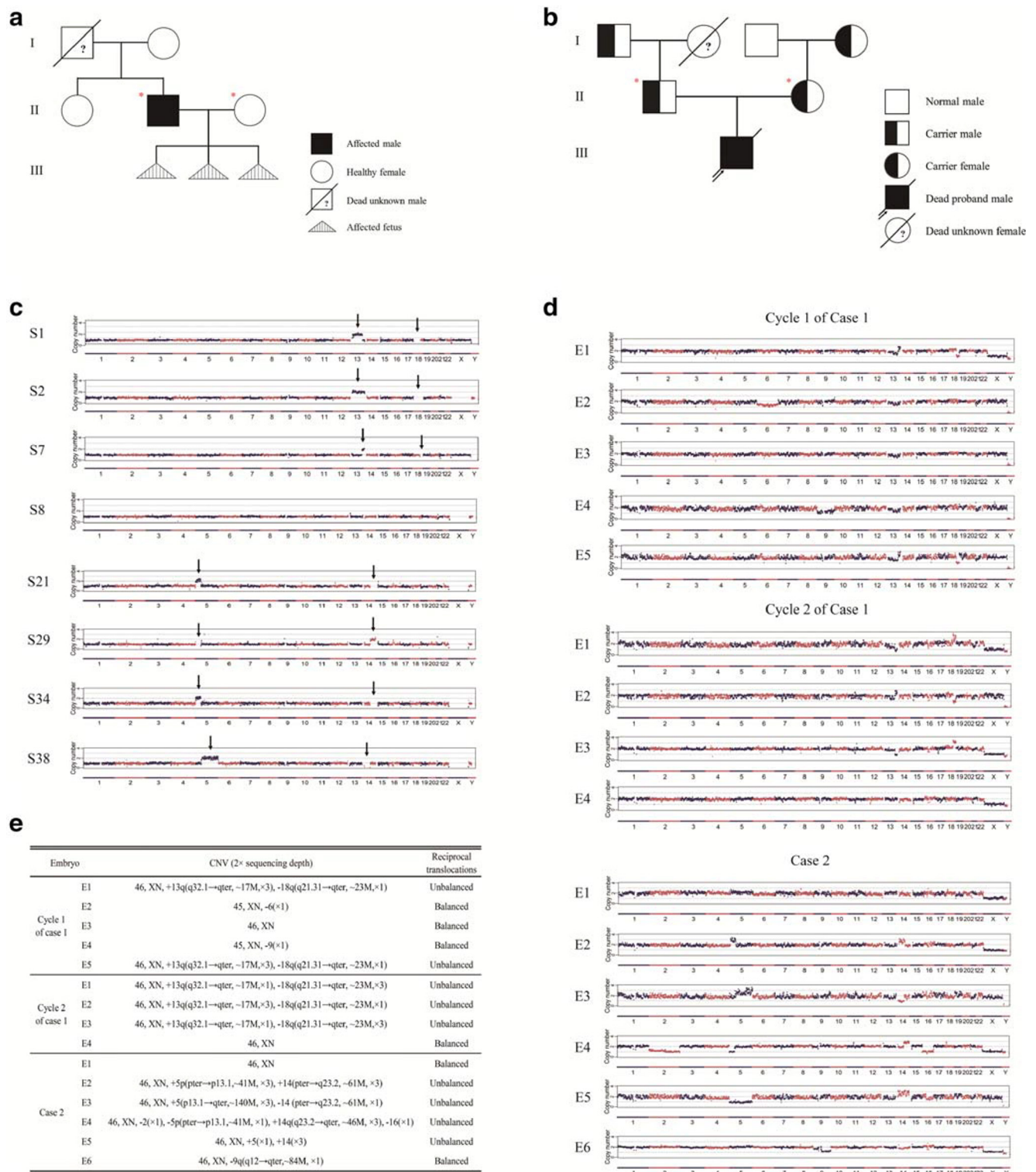
### Blastocyst biopsy and single sperm collection

In case 1, the couple underwent two oocyte retrieval cycles. Five embryos in cycle 1 and four embryos in cycle 2 were biopsied on day 5 or 6 after fertilization at the blastocyst stage to collect trophectoderm cells for later detection. In case 2, the couple underwent one oocyte retrieval cycle. In total, six embryos at the blastocyst stage were biopsied. After washing in PBS (with 0.1% HSA), the biopsied trophectoderm (TE) cells were transferred to a 0.2-mL PCR tube filled with 5  $\mu$ L lysis buffer.

Semen quality was firstly assessed by sperm concentration, motility, and morphology. Higher motile sperms ( $n = 10$ ) with morphological integrity were captured and collected individually into ten separate PCR tubes for next whole genome amplification (WGA), then a portion of the WGA products was used for mutant allele analysis by PCR-Sanger sequencing. The rest of the WGA products from two sperms carrying mutant alleles and two sperm carrying wild-type alleles were selected for next NGS and haplotype analysis.

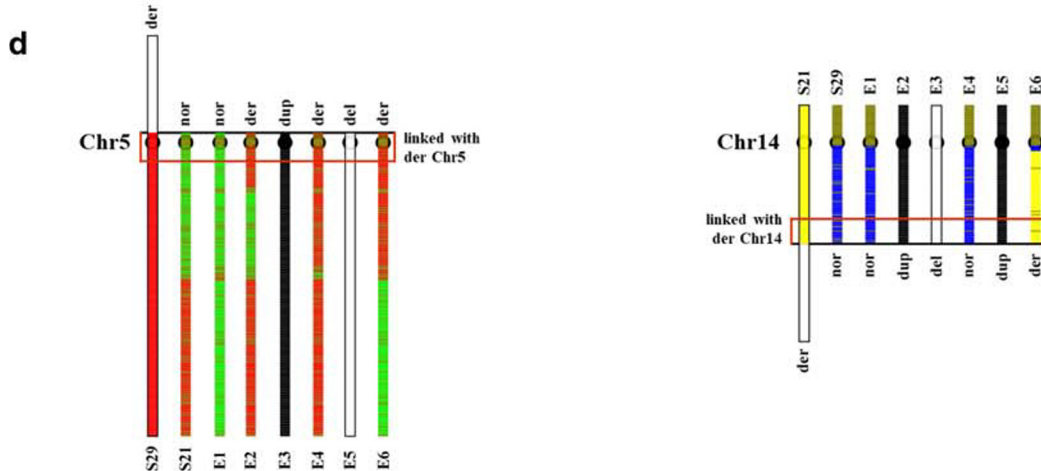
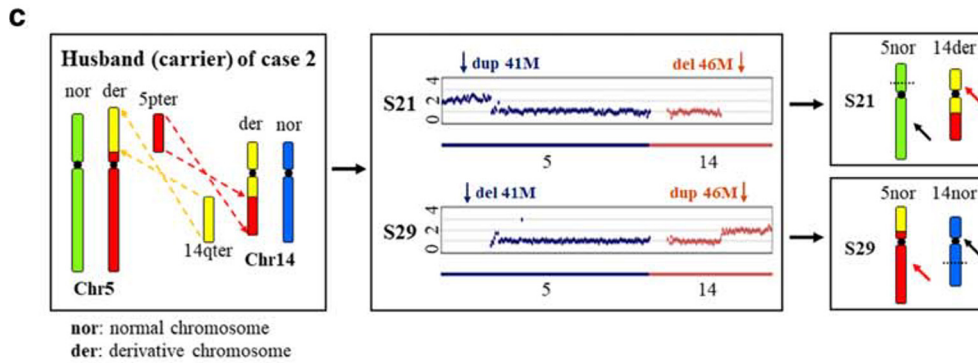
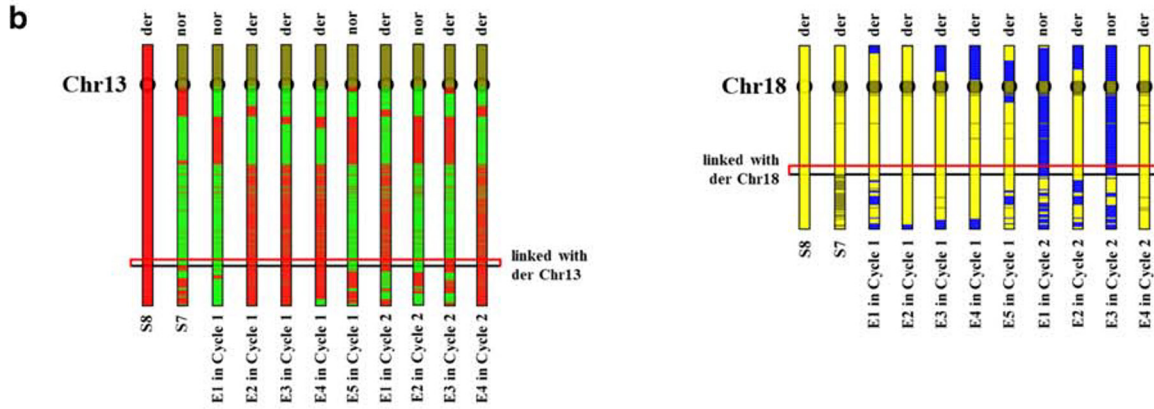
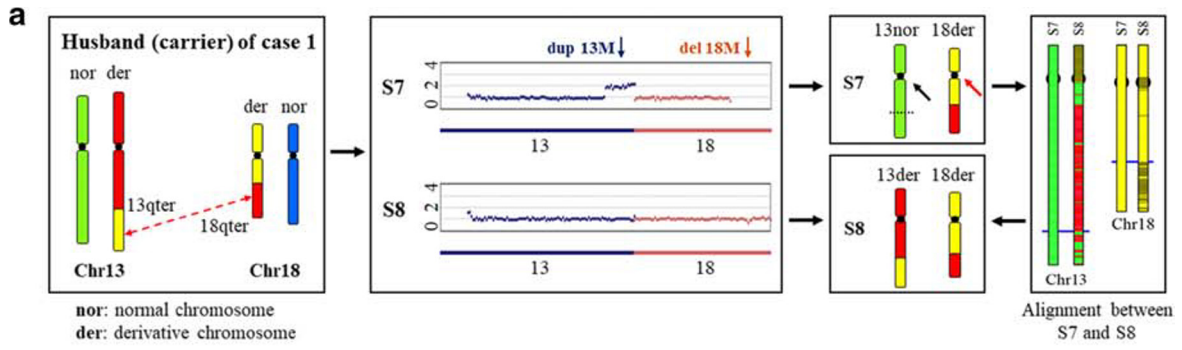
### WGA, specific PCR, and NGS

We used a novel PGD strategy based on single sperm linkage analysis and MARSALA technology to inspect chromosomal abnormalities and mutation sites, and to perform linkage analysis including sperm linkage for reciprocal translocation and single gene disorders diagnosis at the same time [31]. The whole genome amplification (WGA) of lysed TE cells or sperm cells was performed using the commercial MALBAC amplification kit according to the protocols (Yikon Genomics Inc.). Then we amplified *NF2* mutation loci (*NF2*, c.1574 + 2 T > C) in case 1, and two different sites (EX7 + 6 and



**Fig. 1** Chromosome aneuploidy analysis of embryos. **a** Pedigrees of the NF type II family. Filled symbols represent affected patient of NF type II; open symbols represent wild-type individuals with respect to NF type II. Circles and squares indicate females and males, respectively. Triangles with vertical lines mean induced labor fetus with chromosome 13 and 18 abnormality. Diagonal lines represent deceased individuals and question mark means lack of related gene detection. Couple indicated by red asterisks asked for PGD treatment. **b** Pedigrees of this SMA combined reciprocal translocation family. Filled symbols represent affected patient of SMA; half-filled symbols represent SMA carriers; open symbols

represent wild-type individuals with respect to SMA. Circles and squares indicate females and males, respectively. The arrow indicates the affected proband. Diagonal lines represent deceased individuals and question mark means lack of SMA-related gene detection. Couple indicated by red asterisks asked for PGD treatment. **c** CNVs of 4 randomly selected embryos each family at 2 × depth. The arrow indicates the abnormal segment of Chr 13 and Chr 18 of case 1 and Chr 5 and Chr 14 of case 2. **d** CNVs of the 15 embryos from the two cases at low sequencing depth (2 ×) of NGS. **e** CNV and reciprocal translocation summary of the 15 embryos



**Fig. 2** Identification of normal embryos from reciprocal translocation carrier embryos. **a** The reciprocal translocation schematic illustration of the husband between Chr 13 and Chr 18 and haplotype of two typical sperms deduced from their CNVs of case 1. S7, which inherited derivative Chr18, is regarded as a proband for analysis of Chr18. S8, which inherited derivative Chr13 and Chr18 deducing from its CNV and comparison with S7, is regarded as a proband for analysis of Chr13 and Chr18. nor, normal chromosome around breakpoint; der, derivative chromosome around breakpoint; pter, chromosome p arm terminal; qter, chromosome q arm terminal. Black arrows indicate normal chromosomes. Red arrows indicate derivative chromosomes. Dotted lines represent breakpoint. **b** Haplotype of paternal inherited chromosomes for each embryo of case 1. Color differences (red vs. green, yellow vs. blue) of the region around breakpoint positions (red boxes indicated) were used to determine the inherited chromosomes. Red and green represent derivative Chr13 and normal Chr13, respectively. Yellow and blue represent derivative Chr18 and normal Chr18, respectively. **c** The reciprocal translocation schematic illustration of the husband between Chr 5 and Chr 14 and haplotype of two typical sperms deduced from their CNVs of case 2. S29, which inherited derivative Chr5, is regarded as a proband for analysis of Chr5. S21, which inherited derivative Chr14, is regarded as a proband for analysis of Chr14. nor, normal chromosome around breakpoint; der, derivative chromosome around breakpoint; pter, chromosome p arm terminal; qter, chromosome q arm terminal. Black arrows indicate normal chromosomes. Red arrows indicate derivative chromosomes. Dotted lines represent breakpoint. **d** Haplotype of paternal inherited chromosomes for each embryo of case 2. Color differences (red vs. green, yellow vs. blue) of the region around breakpoint positions (red boxes indicated) were used to determine the inherited chromosomes. Red and green represent derivative Chr5 and normal Chr5, respectively. Yellow and blue represent derivative Chr14 and normal Chr14, respectively. White regions indicate deletion of this part of chromosome. Dup, duplication; del, deletion

IVS7 + 236) in case 2 that vary between *SMN1* and *SMN2*, from the WGA product, using specific primers for Sanger sequencing and NGS-based quantities analysis. Specific PCR was performed at 98 °C for 30 s, 30 cycles of 15 s at 98 °C, 30 s at 58 °C, and 30 s at 72 °C, and an additional 2 min at 72 °C using tag enzyme (NEB Inc.). This PCR product was mixed with the MALBAC product (0.5–2% of MALBAC product), and the mixture was used to construct a library using the NEBNext Ultra II DNA library Prep kit (New England Biolabs, Inc.). Copy number variations (CNVs) were measured accurately at a low depth (2×) by using the read depth method [30, 31]. The sequencing was performed on an Illumina HiSeq X Ten platform.

### Linkage analysis by MARSALA

To identify normal embryos from reciprocal translocation carrier embryos, we separated Chr13 (Chr5) and Chr18 (Chr14) haplotyping based on typical sperms' CNVs using SNPs (Fig. 2b). For the NF type II family, we separated the *NF2* wild-type allele and mutation allele based on SNPs of typical sperms S7 and S8 (Fig. 3b). Eight SNPs within 1 Mb upstream and downstream of the *NF2* mutation loci were rechecked by Sanger sequencing (Fig. 3c). For the SMA

family, we separated haplotypes around the *SMN1* gene on Chr5 based on typical sperms and maternal family members' SNPs information for identification of the mutant allele (Fig. 4b and c). We carefully checked SNP positions within 3 Mb upstream and downstream of the *SMN1* gene, and 13 SNPs were rechecked by Sanger sequencing (Fig. 4d). For example, if the father is heterozygous C/G and the mother is homozygous C/C in one SNP marker, while the sperm inherited the mutant allele C, we can deduce that the base of C from the father is linked with the mutant allele, and the base of G is linked with the normal allele.

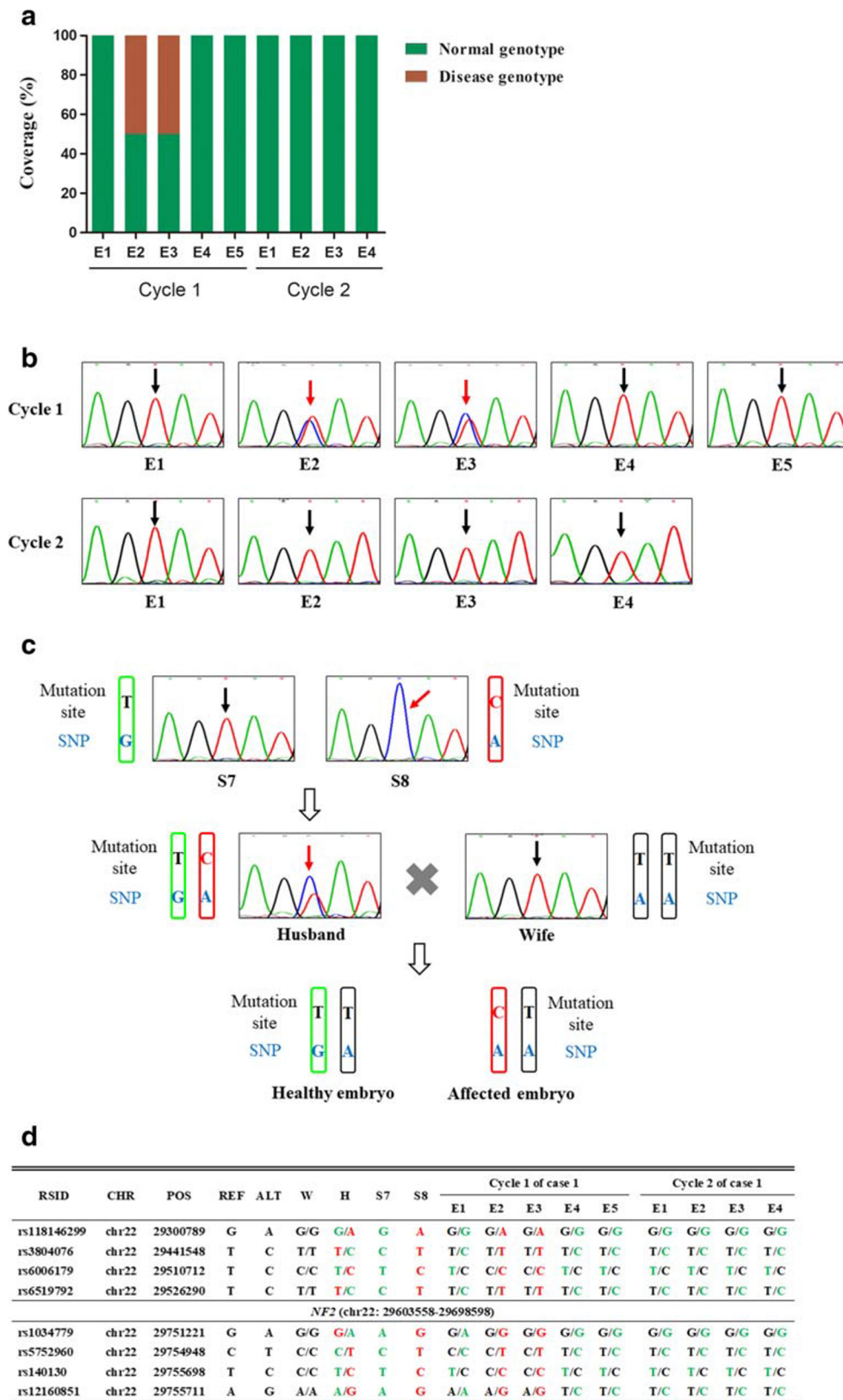
### NGS-based quantitative analysis

This analysis was designed for the SMA family to verify the copy number of *SMN1*. We incubated equally 8 ng MALBAC product of each biopsied sample for specific PCR, and the PCR product was mixed with the MALBAC product (0.5–2% of MALBAC product) in a specific quantity. We stringently controlled quantities of each sample in every step to ensure that sequencing quantities of each sample were equal, and that the amplification efficiency of *SMN1* and *SMN2* are approximately identical. NGS sequencing can distinguish and count *SMN1* fragments and *SMN2* fragments. From the above, we can directly obtain copy numbers of *SMN1* and *SMN2* through normalizing and comparing (Fig. 4a).

## Results

### Sperm detection and chromosome aneuploidy analysis

In case 1, after the NF type II family had three abnormal pregnancies, we tested the couple for chromosomal abnormalities. It was confirmed that the husband was a carrier of a reciprocal translocation between Chr13 and Chr18 [t(13; 18)(q32; q21.3)], as well as the *NF2* gene pathogenic variant (*NF2*, c.1574 + 2 T > C) (Fig. 1a). In case 2, both husband and wife carried pathogenic variants (*SMN1*, exons 7 and 8 deletion) and their child was fatally affected with SMA (Fig. 1b). Lacking the genetic information of an essential family member for linkage analysis (Fig. 1a, b), single sperm was used for paternal linkage analysis and to assist in identification of pathogenic variant sites. Before undergoing the PGD cycle, four sperms from each case (S1, S2, S7, and S8 in case 1; and S21, S29, S34, and S38 in case 2) were selected and amplified separately, followed by pathogenic variant detection and NGS to distinguish mutant alleles from wild-type alleles in each family. S1 and S7 each carry the wild-type allele by amplification of *NF2* mutation site, whereas S2 and S8 each carry the pathogenic variant allele (Fig. 1c). S29 carries the



disease-causing allele by amplification of the *SMN1* and *SMN2* different site 2, while S21 carries the wild-type allele by amplification of the *SMN1* and *SMN2* different site 1.

Unexpectedly, we observed chromosome aneuploidy with Chr5 and Chr14 in each sperm from case 2 (Fig. 1c). Based on this observation, we speculated that the husband could be a

◀ **Fig. 3** Diagnosis and confirmation of NF type II of nine embryos from two cycles. **a** Result of NGS sequencing the targeted mutation site in the *NF2* gene. E2, and E3 of cycle 1 are affected embryos. **b** Sanger sequencing of *NF2* mutation site of nine embryos. Black arrows indicate wild-type embryos. Red arrows indicate affected embryos. **c** Paternal SNP analysis schematic depending on normal and mutant sperms. The black “T” means S7 carrying wild-type allele and the blue “G” indicates the base associated with wild-type allele of *NF2* gene. The red “C” means S8 carrying mutation allele and the blue “A” indicates the base linked with mutation allele of *NF2* gene. Embryo carrying blue “G” means it inherited wild-type allele while embryo carrying blue “A” means it inherited mutation allele. **d** Results of linkage analyses of the nine embryos. Four upstream and four downstream SNP markers were selected to identify the disease-carrying allele in each embryo. RSID, reference SNP cluster ID; CHR, chromosome number; POS, genomic position; REF, reference allele of the SNPs; ALT, alternative allele of the SNPs; W, wife; H, husband

reciprocal translocation carrier. To confirm this speculation, we performed FISH (fluorescence in situ hybridization), and the father was diagnosed as a carrier of a reciprocal translocation between Chr5 and Chr14 [t (5; 14) (p15; q32)]. Both of these exceptional cases involved a single gene disorder and reciprocal translocation, meanwhile both cases lack essential family members required for conventional paternal linkage analysis. To perform PGD for these complicated cases, we employed the new testing strategy mentioned above.

Chromosome CNV analysis of fifteen embryos from 3 cycles showed that E3 in cycle 1 of case 1, E4 in cycle 2 of case 1, and E1 in case 2 were balanced, and no other chromosomal abnormalities were observed, whereas chromosomal aneuploidies were observed in other embryos. The majority of the abnormalities occurred in translocation chromosomes, including E1 (unbalanced) and E5 (unbalanced) in cycle 1 of case 1; E1 (unbalanced), E2 (unbalanced), and E3 (unbalanced) in cycle 2 of case 1; and E2 (unbalanced), E3 (unbalanced), E4 (unbalanced and monosomy 2, 16), and E5 (unbalanced) in case 2. Meanwhile, other chromosomal abnormalities occurred, including E2 (monosomy 6) and E4 (monosomy 9) in cycle 1 of case 1 and E6 (deletion in part of chromosome 9) in case 2 (Fig. 1d, e).

### Distinguishing wild-type embryos from reciprocal translocation carrier embryos

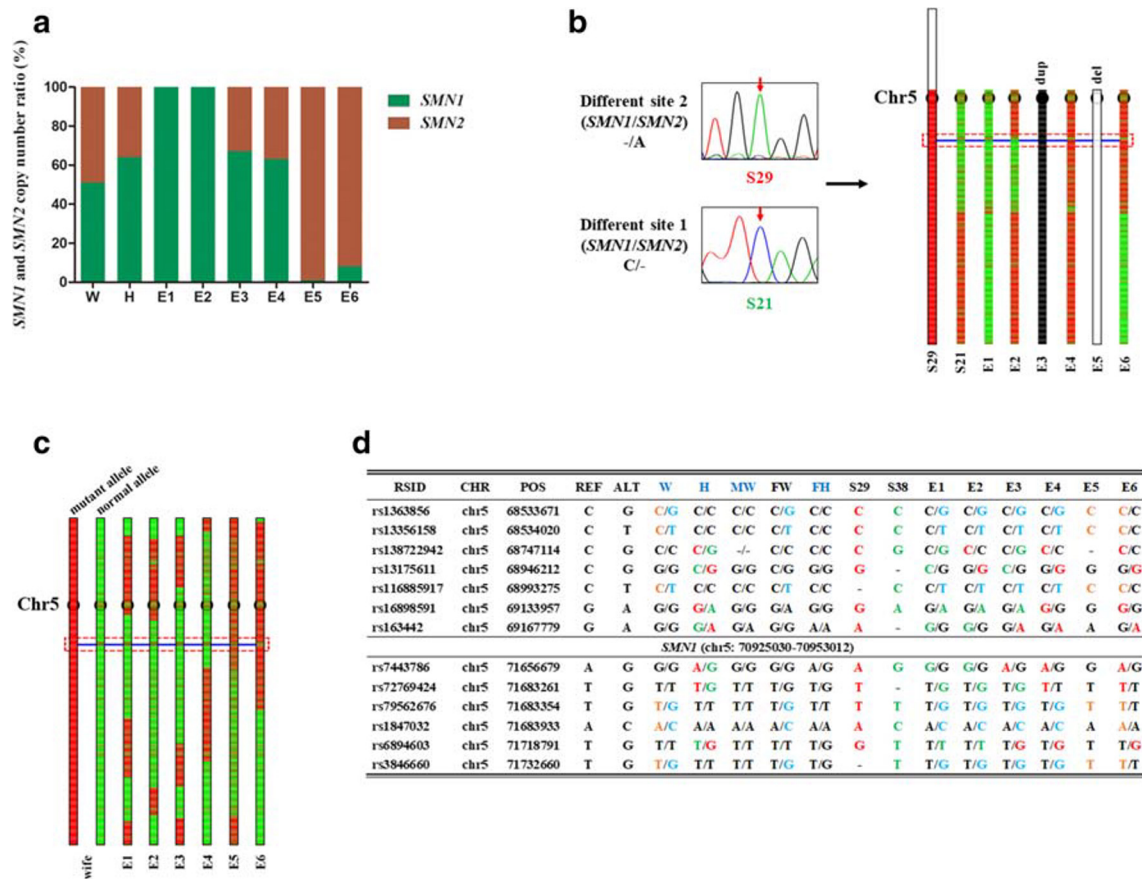
Through CNV analysis, embryos with abnormal chromosome copy numbers were identified, but it was difficult to distinguish between wild-type and translocation carrier embryos. To overcome this challenge, we chose an additional chromosome haplotyping method based on single sperm CNV analysis [32]. Two sperms were selected for haplotyping analyses that inherit different types of translocation chromosomes caused by reciprocal translocation in each family. In case 1, Fig. 2a illustrates the translocation events between the Chr13 q arm and Chr18 q arm of the male carrier. According to the CNV result of S7, we can deduce that S7 inherited normal Chr13

and derivative Chr18. Other than S7, S8 had no obvious copy number abnormality, so we sought to deduce whether S8 carried normal Chr13 and Chr18 or derivative Chr13 and Chr18. Compared with S7, we inferred that S8 carried derivative Chr13 and Chr18 (Fig. 2a). From CNV analysis, we know that Chr13 and Chr18 of E2, E3, and E4 of cycle 1, and E4 of cycle 2 were amphidiploid (Fig. 1e). Haplotypes of embryos and alignment between embryos and S8 told us that all four embryos were reciprocal translocation carriers (Fig. 2b). Using E4 of cycle 2 as an example, which is consistent with S8 in the red region linked with derivative Chr13 and Chr18, we deduced that S8 carried derivative Chr13 and Chr18. Figure 2c illustrates the translocation events between the Chr5 p arm and Chr14 q arm of the male carrier of case 2. Through sperm CNV and translocation patterns, we can deduce which chromosomes were present in the sperm. S21 inherited normal Chr5 and derivative Chr14, and can provide partial (yellow region indicated by red arrow) haplotype linkage information of derivative Chr14. Likewise, S29 inherited derivative Chr5 and normal Chr14, and can provide partial (red region indicated by red arrow) haplotype linkage information of derivative Chr5. Paternal and maternal haplotypes of the embryos were separated depending on sperm haplotype (Fig. 2d). Based on the chromosome aneuploidy analysis, we know that Chr5 and Chr14 of E1 and E6 were amphidiploid, as shown in Fig. 2d. For example, also as shown in Fig. 2d, we took part of S29 (red region) as derivative Chr5 proband, and E6 is consistent with S29, which allowed us to deduce that E6 inherited derivative Chr5. E6 is consistent with S21 in the red region linked with derivative Chr14, which allowed us to deduce that E6 inherited derivative Chr14. Thus, only E1 inherited normal Chr5 and Chr14 that were inconsistent with S29 and S21.

Using chromosome haplotyping analysis, we can accurately determine that E1 of case 2 is wild-type, while the other five embryos analyzed above are carriers of the reciprocal translocation.

### Diagnosis of single gene disorders

Mutation site detection and SNP linkage analysis were used together to ensure the accuracy of the diagnosis of the NF type II family (case 1). Both NGS sequencing (Fig. 3a) and Sanger sequencing (Fig. 3b) were performed after specific PCR amplification for the *NF2* pathogenic variant (*NF2*, c.1574 + 2 T > C) to determine affected embryos. E2 and E3 of cycle 1 were identified as carrying the paternal pathogenic variant, whereas the other seven embryos of this family were identified as being free of NF type II. To avoid allele drop-out, single sperm SNP linkage analysis was established to verify whether pathogenic variant alleles were present in the embryos. S8 carried pathogenic variant alleles which could be taken as the proband, while S7 inherited wild-type alleles which could be taken as the normal control (Fig. 3c). Eight SNP



**Fig. 4** Diagnosis and confirmation of SMA of six embryos. **a** NGS results of copy number ratio of *SMN1* and *SMN2* by sequencing different site 1. E5 and E6 are affected embryos while the other embryos have at least one copy of *SMN1*. W, wife; H, husband. **b** Haplotype of six embryos for paternal linkage analysis depending on S29 (disease-carrying allele) and S21 (normal allele). E1 and E2 inherited the normal allele, while E4 and E6 inherited the disease-carrying allele. E5 lacked the paternal chromosome 5 q arm. E3 inherited both the normal allele and disease-carrying allele from the father. **c** Haplotype of six embryos for maternal linkage analysis depending on mother’s parents. The mother’s mutant allele was inherited from her mother and normal allele

from her father. E1, E2, E3, and E4 inherited the normal allele from the mother, while E5 and E6 inherited the disease-carrying allele. **d** Results of linkage analyses of the six embryos based on SNP. Seven upstream and six downstream SNP markers were selected to identify the disease-carrying allele in each embryo. RSID, reference SNP cluster ID; CHR, chromosome number; POS, genomic position; REF, reference allele of the SNPs; ALT, alternative allele of the SNPs; W, wife; H, husband; MW, mother of the wife; FW, father of the wife; FH, father of the wife. Adult carriers labeled in dark blue. - represents the alleles that are not covered by single-cell low-depth sequencing and Sanger sequencing

markers (within 1 Mb around *NF2*) were selected from NGS linkage analysis and confirmed by Sanger sequencing to illustrate single sperm linkage results (Fig. 3d). In concordance with the mutation site detection analysis, the linkage analysis verified that only E2 and E3 from cycle 1 were affected with NF type II.

Compared with the NF type II family, it is more difficult to diagnose SMA in the embryos of the SMA family (case 2). Because *SMN2* is highly homologous to *SMN1*, direct detection of mutations in *SMN1* is difficult, especially in embryos. Therefore, to improve diagnostic accuracy, we used three independent methods to confirm this diagnosis. Table 1 shows Sanger sequencing results at two different loci that vary between *SMN1* and *SMN2*, which can distinguish affected embryos from unaffected embryos. E5 and E6 lost two copies of *SMN1* exons 7 and 8, and

were thus diagnosed as affected embryos, whereas the other embryos tested had at least one copy of *SMN1* exons 7 and 8. Furthermore, we examined the different site 1 between *SMN1* and *SMN2* of the six embryos. Certain

**Table 1** Sanger sequencing results at two different sites varying between *SMN1* and *SMN2* for six embryos

Embryo	Different site 1 ( <i>SMN1/SMN2</i> )	Different site 2 ( <i>SMN1/SMN2</i> )
E1	C/-	G/-
E2	C/-	G/-
E3	C/T	G/A
E4	C/T	G/A
E5	-/T	-/A
E6	-/T	-/A



sequences containing different sequences between *SMN1* and *SMN2* were amplified using specific primers and detected by NGS. Through this detection method, we reconfirmed that E5 and E6 are affected embryos, whereas E1 and E2 are wild-type embryos free of *SMN1* mutation (Fig. 4a, Table S1, and Fig. S1C). In addition to analyzing embryos using these two methods, sperm linkage for paternal mutation analysis and pedigree linkage for maternal mutation analysis further confirmed the accuracy of the diagnosis. As shown in Fig. 4b, S29 was determined to be the proband carrying the mutant allele by Sanger sequencing of the different site 2, while S21 carried the normal allele by Sanger sequencing of the different site 1. From Fig. 4b, we can deduce that E1 and E2 inherited the normal allele from the father, while E4 and E6 inherited the mutant allele from the father. The lack of chromosome 5q arm in E5 was from the father. E3 was more complicated, inheriting both a normal allele and a disease-carrying allele from the father. Compared to analyzing the paternal pathogenic variant-carrying allele, the maternal pathogenic variant-carrying allele was much more straightforward. E5 and E6 inherited the pathogenic variant-carrying allele, whereas the other embryos inherited the normal allele from the mother (Fig. 4c). To be more precise, seven SNPs (within 2.4 Mb upstream of *SMN1*) and six SNPs (within 0.8 Mb downstream of *SMN1*) listed in the NGS linkage analysis results were confirmed by Sanger sequencing (Fig. 4d). E1 and E2 inherited normal alleles from both parents, whereas E6 inherited pathogenic variant-carrying alleles from both parents. Both haplotyping analysis (Fig. 4b) and SNP analysis (Fig. 4d) revealed that there is paternal chromosome exchange at about 2 Mb upstream of *SMN1* of E2. E5 only inherited the pathogenic variant-carrying allele from the mother. It is worth noting that fraternal linkage analysis of E3 is ambiguous, and 4 SNPs indicated that it inherited the normal allele, while 3 SNPs indicated that it inherited the disease-carrying allele. This is likely because E3 carried genetic materials from both chromosome 5 of the paternal allele.

### Summary of the detection and clinical outcome

Chromosome aneuploidy analysis, reciprocal translocation detection, and single gene disease diagnosis revealed that E4 in cycle 2 of case 1, and E1 in case 2 were suitable for embryo transfer because they were unaffected by the monogenic disorders tested, and displayed no significant chromosomal alterations (specifically, E1 in case 2 was free of the reciprocal translocation) (Table 2). All other embryos were unsuitable for transfer due to aneuploidy and/or being affected by single gene disorders. Furthermore, E2 and E3 in cycle 1 of case 1 inherited paternal pathogenic variants, and E5 and E6 in case 2

also carried *SMN1* pathogenic variants from both parents (Table 2).

E1 in case 2 was transplanted, but unfortunately, the embryo failed to implant. In case 1 cycle 2, the E4 that inherited paternal wild-type *NF2* allele and the chromosome reciprocal translocation was abandoned upon the couple's request, as they preferred to move on to the next cycle.

## Discussion

### Unique features of these families and comparison of our new PGD strategy with other methods

In the cases of the two families in this study, there were obstacles in paternal linkage analysis due to lacking information from essential family members. Additionally, both reciprocal translocations from the husbands in each couple, as well as the *NF2* pathogenic variant in the husband of case 1, are de novo. In the NF type II family, we speculate that the husband carries a de novo pathogenic variant of *NF2*, which is not found in other living family members, and was not symptomatic in his father during his lifetime. Meanwhile, in the SMA family, it was unknown whether the husband inherited the *SMN1* pathogenic variant from his father or mother (his mother passed away), since its high carrier and de novo frequency. Detection of *SMN1* copy number in PGD relies on linkage analysis to provide sufficient evidence to eliminate *SMN2*'s interference, and successfully distinguish normal embryos from carrier embryos [13, 22, 25]. Without this information, the accuracy of the diagnosis would have been compromised. To overcome these difficulties, and to increase the accuracy of diagnosis, additional information about CNVs, pathogenic variants, SNPs, and reciprocal translocation analysis with NGS was necessary. Since invasive procedures were required to retrieve the biopsied trophoctoderm (TE) cells, our novel approach allows us to collect as much information as we can without using substantial amounts of starting material, which conventional FISH and PCR-based direct mutation detection were not able to do [14, 17]. The other typical approach used in PGD, array-CGH, cannot provide information on single gene mutations, and fails to detect extra or missing fragments smaller than 6 Mb [14].

To improve the accuracy of diagnosis, we established a new method combining sperm linkage with NGS-based quantitative analysis on the basis of MARSALA technology. CNVs, SNVs, and SNP information were simultaneously obtained from one NGS run [11, 17, 31]. In particular, for single gene mutation detection, single sperm linkage can help distinguish normal alleles from mutant alleles. NGS-based analysis identifies the specific sites different between *SMN1* and *SMN2*; it can also provide copy number information for exons

**Table 2** Summary of PGD results for fifteen embryos

Embryo	CNV	Reciprocal translocation	Diagnosis for monogenic disorder	
Case 1		Between Chr13 and Chr18	NF type II (paternal mutation)	
Cycle 1	E1	Aneuploid	Unbalanced	No
	E2	Aneuploid	Carrier	Yes
	E3	Euploid	Carrier	Yes
	E4	Aneuploid	Carrier	No
	E5	Aneuploid	Unbalanced	No
Cycle 2	E1	Aneuploid	Unbalanced	No
	E2	Aneuploid	Unbalanced	No
	E3	Aneuploid	Unbalanced	No
	E4	<i>Euploid</i>	<i>Carrier</i>	<i>No</i>
Case 2		Between Chr5 and Chr14	SMA	
			Maternal mutation	Paternal mutation
<i>E1</i>	<i>Euploid</i>	<i>Normal</i>	<i>No</i>	<i>No</i>
E2	Aneuploid	Unbalanced	No	No
E3	Aneuploid	Unbalanced	No	Yes
E4	Aneuploid	Unbalanced	No	Yes
E5	Aneuploid	Unbalanced	Yes	–
E6	Aneuploid	Carrier	Yes	Yes

E4 in cycle 2 of case 1, and E1 in case 2 were suitable for embryo transfer because they were unaffected by the monogenic disorders tested, and displayed no significant chromosomal alternations

7 and 8 in *SMN1*, which is undetectable by karyomapping approaches [27]. NGS followed by two site-specific PCRs can help evaluate copy numbers of *SMN1* (exons 7 and 8) using the *SMN2* homologous fragment as the reference. This method has never been reported before on embryonic single-cell sequencing analysis. To detect reciprocal translocations in embryonic single cells, we relied on chromosome haplotype analysis to differentiate wild-type embryos from carrier embryos, a method which can achieve high accuracy by deep sequencing [8, 32]. The flexible mutation sites used for PCR design and variable sequencing depth depending on clinical demand make this method more widely applicable than other methods such as array-CGH and karyomapping, especially for complicated clinical cases described here and for other monogenic disorder cases difficult to detect [6, 12, 17, 18].

### The new NGS-based quantitative analysis can be used for PGD in other cases beyond SMA

SMA represents a challenging monogenic disorder for PGD because the pathogenic variant cannot be detected directly in embryos, not only due to the mutation type (exon deletion) but also the existence of a highly homologous gene. *SMN1* and *SMN2* are highly homologous, and this new NGS-based quantitative method could determine the copy numbers of these two homologous genes in one PCR reaction using the same primers. To deduce the copy number of *SMN1*, the copy number of *SMN2* is used as a reference. This approach needs further refinement in the future, as the number of *SMN2* copies

can be variable [3, 16]. In this case, the copy numbers of *SMN1* and *SMN2* of family members were identified by MLPA (Multiplex Ligation-dependent Probe Amplification), allowing us to deduce the precise gene copy number in embryos by our new NGS-based quantitative method. Here, we deduced copy number variation and illustrated the allele inheritance using MLPA, sperm analysis, and NGS-based quantitative analysis results (Fig. S1 and Table S1). In Fig. S1A, we illustrated the MLPA results of the family members. MLPA detection, combined with sperm haplotyping analysis, constituted the inheritance origin of the alleles of the embryos (Fig. S1B). In addition, SNP-linkage analysis (Fig. 4b and c) and NGS-based quantitative analysis (Fig. 4a) were used to identify copy numbers of *SMN1* and *SMN2* and the inheritance map of the six embryos in case 2 (Fig. S1C). This new NGS-based quantitative analysis strategy is a reliable approach for detection of copy number in PGD. It is extraordinarily powerful in the evaluation of monogenic diseases involving copy number in embryos.

### Our advanced MARSALA method is efficient and accurate for reciprocal translocation diagnosis

For the first time, the advanced MARSALA approach is reported as a promising diagnostic method for complicated PGD cases containing reciprocal translocation and single gene disorder. Compared with array-CGH, this method is more efficient, as we obtained copy number in higher resolution and even at the single gene level, with one DNA library

preparation and one next-generation sequencing run. Furthermore, it is more accurate than traditional methods, since it uses several lines of evidence for the two complicated cases.

In case 2, the husband has a reciprocal translocation between Chr5 and Chr14, which is particularly relevant as the *SMN1* gene is located on Chr5. We found that this derivative Chr5 serves as a marker for the mutant allele of *SMN1* deletion mutation in the husband of case 2. From S21 and S29 CNV analysis and *SMN1* specific detection results, it was clearly shown that the *SMN1* deletion allele is linked with derivative Chr5, and the *SMN1* wild-type allele is linked with normal Chr5 (Fig. S1B). Thus, we deduced that embryos that inherited normal Chr5 inherited the *SMN1* wild-type allele (ignoring chromosome exchange). Following this simple ratiocination with combined information from the sperm and embryos, E1, E4, and E6 in case 2 could be diagnosed (Fig. S1C). In conclusion, we have shown through this representative PGD case that with a modified MARSALA and NGS detection strategy combined with haplotype information from the sperm, one can provide accurate diagnoses for couples with complicated chromosomal abnormality changes (such as reciprocal translocation) and monogenetic diseases (such as NF2 and SMA).

### Limitations of this novel strategy

There are a few limitations of this novel strategy. Firstly, we need single sperm DNA analysis to assist embryo diagnosis. NGS of multiple single sperm in separate tubes will increase costs. Secondly, this novel strategy is more appropriate for male patients or carriers. For female patient or carriers, polar bodies taken as probands are needed. If there are no sufficient matured oocytes and embryos, it is difficult to perform genetic diagnosis using this approach.

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