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Identification of a cryptic unbalanced translocation Der(22)t(12;22)(q24.33;q13.33) in a large Chinese family with Phelan-McDermid syndrome by nanopore sequencing

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To explore the genetic cause of a four-generation severe intellectual disability in a Chinese family using nanopore sequencing and to provide genetic counseling and reproductive guidance for family members. Multiple genetic analyses of the proband and family members were performed, including chromosome karyotype analysis, whole exome sequencing, nanopore sequencing, PCR amplification, and Sanger sequencing. The results of G-binding karyotyping, CGG repeats for *FMR1*, GGC repeats for *NOTCH2NCL*, and trio-whole-exome sequencing were negative for the proband and his parents. Nanopore sequencing showed that the proband carried 12q24.33 microduplication (3.26 Mb) and 22q13.33 microdeletion (1.5 Mb). According to the guidelines of the American Society for Medical Genetics and Genomics (ACMG), the 22q13.33 microdeletion was classified as pathogenic, whereas the 12q24.33 microduplication was classified as a variant of uncertain significance (VUS). The precise karyotype and location of chromosomal breakpoints in the patient and family members were determined through PCR. According to the results of Sanger sequencing, a cryptic balanced translocation was detected in the proband's father. Additionally, informative SNPs were identified near the breakpoints for preimplantation genetic testing for structure rearrangement (PGT-SR) treatment by nanopore sequencing. We identified a cryptic unbalanced translocation in a large Chinese family with Phelan-McDermid syndrome (22q13.33 deletion syndrome) by nanopore sequencing. Nanopore sequencing can be a powerful tool for the genetic diagnosis of unexplained intellectual disability and the detection of precise breakpoints of chromosomal rearrangement in PGT-SR treatment.

Keywords Phelan-McDermid syndrome, Nanopore sequencing, Cryptic balanced translocation, 22q13.33 deletion

Phelan McDermid syndrome (PMS, OMIM #606232), also known as 22q13.3 deletion syndrome, is a complex neurodevelopmental disorder characterized by intellectual disability, absent or severely delayed speech, neonatal hypotonia and autistic spectrum disorder (ASD)^{1–3}. Neurological and behavioral phenotypes are usually severe, and most PMS patients have moderate to profound intellectual disabilities⁴. Dysmorphic features are usually mild and include long eyelashes, large ears, bulbous nose, large fleshy hands, and dysplastic toenails⁵. Additional features include seizures, motor deficits, renal malformations, decreased perspiration, and recurrent infections⁵.

Identifying the molecular genetic cause of PMS is important for prescribing appropriate treatments, clinical management, genetic counseling, and reproductive options for subsequent pregnancies⁶. Owing to recent advances in technology, especially microarrays and next-generation sequencing, the number of recorded PMS

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cases has rapidly increased^{7,8}. The PMS Foundation (www.pmsf.org) reports that there are more than 3,600 individuals with registered genetic disorders in the world. However, choosing the most effective testing method for suspected PMS patients based on clinical phenotypes is extraordinarily difficult and should be explored further.

Recently, third-generation sequencing (TGS), also known as long-read sequencing (LRS), has gradually become an important diagnostic method for genetic diseases⁹. Oxford nanopore sequencing, one of the most popular TGS technologies^{10,11}, can effectively solve several challenges in next-generation sequencing, such as covering highly repetitive and complex regions and overcoming preference bias caused by PCR amplification. Due to its ultra long read length and high throughput, TGS has significant advantages in detecting genomic structural variations, tandem repeat analysis, pseudogene homology analysis, and haplotype analysis. Therefore, it is playing an increasingly important role in genetic disease diagnosis.

In this study, we first applied nanopore sequencing in the genetic diagnosis of PMS patients with unexplained severe intellectual disability and global developmental delay in a four generation Chinese family that had not been diagnosed by several genetic testing methods, including karyotype analysis, repeat tests for *FMR1* and *NOTCH2NLC*, and whole exome sequencing (WES). We believe that nanopore sequencing can facilitate the diagnosis of rare diseases, including PMS, and the provision of effective genetic counseling and treatments for the patient and their family members.

Materials and methods

Samples

This study was approved by the Ethics Committee of Reproductive Medicine of Jiangxi Maternal and Child Health Hospital, and written informed consent was obtained from all participants. Peripheral blood samples of the patient and his family members were obtained for karyotype analysis and DNA extraction. Genome DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Germany), according to the manufacturer's instructions. DNA purity was determined by nanodrop spectrophotometer (Thermo Fisher Scientific, US), and the DNA concentration was measured using a Qubit 3.0 Fluorometer (Invitrogen, US).

Karyotype analysis

Karyotype analysis was performed for the proband and his family members including III:9, III:10 and IV:8. Peripheral blood lymphocytes were routinely collected, cultured and harvested. Metaphase G-banding at 450 band level was performed before conventional cytogenetic analysis by Leica Cytovision system.

Repeat test for *FMR1* and *NOTCH2NLC*.

Repeat-primed polymerase chain reaction (RP-PCR) was used to detect the number of CGG repeats in the *FMR1* and GGC repeats in the gene *NOTCH2NLC* for the proband and his parents (III:9, III:10 and IV:8), as described previously^{12,13}. Briefly, the number of CGG repeats of the gene *FMR1* was determined by GC-rich PCR with *FMR1* primers (Table S1) using AmpliTaq Gold DNA polymerase (Applied Biosystems). The thermal cycling conditions were as follows: denaturation at 98°C for 3 min, 10 cycles of 98°C for 20 s, 65°C for 45 s and 72°C for 3 min, followed by 22 cycles of 98°C for 20 s, 68°C for 3.5 min, and a final extension at 68°C for 10 min. The RP-PCR primer mix for the *NOTCH2NLC* GGC repeat test is also shown in Table S1. RP-PCR conditions included incubation at 98°C for 4 min, 30 cycles of 98°C for 45 s, 60°C for 45 s, 72°C for 4 min, and a final extension at 72°C for 7 min. The PCR products of *FMR1* and *NOTCH2NLC* were analyzed on the ABI 3500 Genetic analyzer (Applied Biosystems).

Trio-whole exome sequencing

Trio-WES was performed using the HiSeq 2500 sequencing platform (Illumina, US) for the proband and his parents (III:9, III:10 and IV:8). Raw fastq data were filtered using FASTQ (V0.23.1) and annotated based on the human reference genome (GRCh37/hg19). The BAM files were generated using the Burrows-Wheeler Aligner (0.7.17-r1194-dirty). The genome analysis toolkit (GATK, V4.20) was used to obtain single nucleotide polymorphisms (SNPs), insertions, and deletions. ANNOVAR and VEP were performed for variation annotations. Pathogenicity classification and interpretation of sequencing variants were performed in accordance with the guidelines of the American College of Medical Genetics and Genomics (ACMG).

Library preparation and nanopore sequencing

A mass of 2 µg of DNA of each proband and his father (III:9 and IV:8) was used as the input material for library preparation of nanopore sequencing. The BluePippin system (Sage Science, US) was used for the size selection of long DNA fragments. The ends of the DNA fragments were repaired and the A-ligation reaction was conducted using the NEBNext Ultra II End Repair/dA-tailings Kit (Cat# E7546, NEB, US). The adapter ligation was performed using the LSK109 Kit and the sizes of the library fragments were quantified by Qubit 3.0 Fluorometer. Sequencing was performed using a PromethION 48 (Oxford Nanopore Technologies, UK).

Copy number variant analysis

The reads were aligned to the reference genome hg19 using minimap2 before structural variants (SVs) with Sniffles were identified, and short tandem repeats were analyzed using RepeatHMM. The copy number of any genomic region could be estimated by counting the number of reads aligned to the consecutive and non-overlapping windows of the genome.

Breakpoint verification by PCR and Sanger sequencing

The PCR primers were designed by the Primer3 software to detect chromosome breakpoints for each sample (II:3, II:4, II:5, III:7, III:8, III:9, III:10, III:12, III:13, III:16, IV:6, IV:8, IV:7, IV:9, IV:10, IV:11). The primer sequences used in this study are shown in Table S1. PCR was performed using 2×Taq Plus Master Mix polymerase (P211-01/02/03, Vazyme). The PCR products were electrophoresed on a 1% agarose gel. Sanger sequencing was performed for the father (III:9) of the proband on an ABI3730XL sequencer (Applied Biosystems).

Haplotype analysis

The MarginPhase method was used to segment the long reads into haplotypes as previously described¹⁴. After identifying candidate structure variants using the combined pipeline, the sequence data were obtained both upstream and downstream of the breakpoint within two megabases. To identify mutations, SNPs/indels were first called using SAMtools mpileup and bcftools. Finally, the haplotype calls were generated using MarginPhase for the proband's father (III:9).

Results

Case description and preliminary analyses

A couple was referred to the reproductive medicine center for reproductive genetic counseling, as they had a son (the proband, IV:8) with severe intellectual disability (Fig. 1A). They also had a non-affected daughter (IV:7). The proband was hospitalized for 28 days due to neonatal jaundice at birth, and exhibited poor immunity during childhood, often experiencing colds and fever. He had nearly normal facial features and limb posture, sparse teeth, poor finger and leg muscle tension, and unstable walking (Fig. 1B). At the age of 11, the patient can pronounce but cannot speak, cannot understand the meaning of speech (can only understand simple words and phrases), suffers from severe intellectual disability, and cannot take care of himself. A detailed family investigation indicated that the other nine family members (III:1, III:4, III:11, III:14, III:15, IV:3, IV:4, IV:6, and IV:9, Fig. 1A) also have similar symptoms.

Karyotype analysis and trio WES

About 5 ml of blood was obtained from the proband and his parents, 2 ml of which was used for G-banding karyotype analysis of 450-band chromosomes according to the standard protocol. No obvious chromosomal abnormalities were observed for the proband and his parents (Fig. 1B). Additionally, the trio-WES of the family

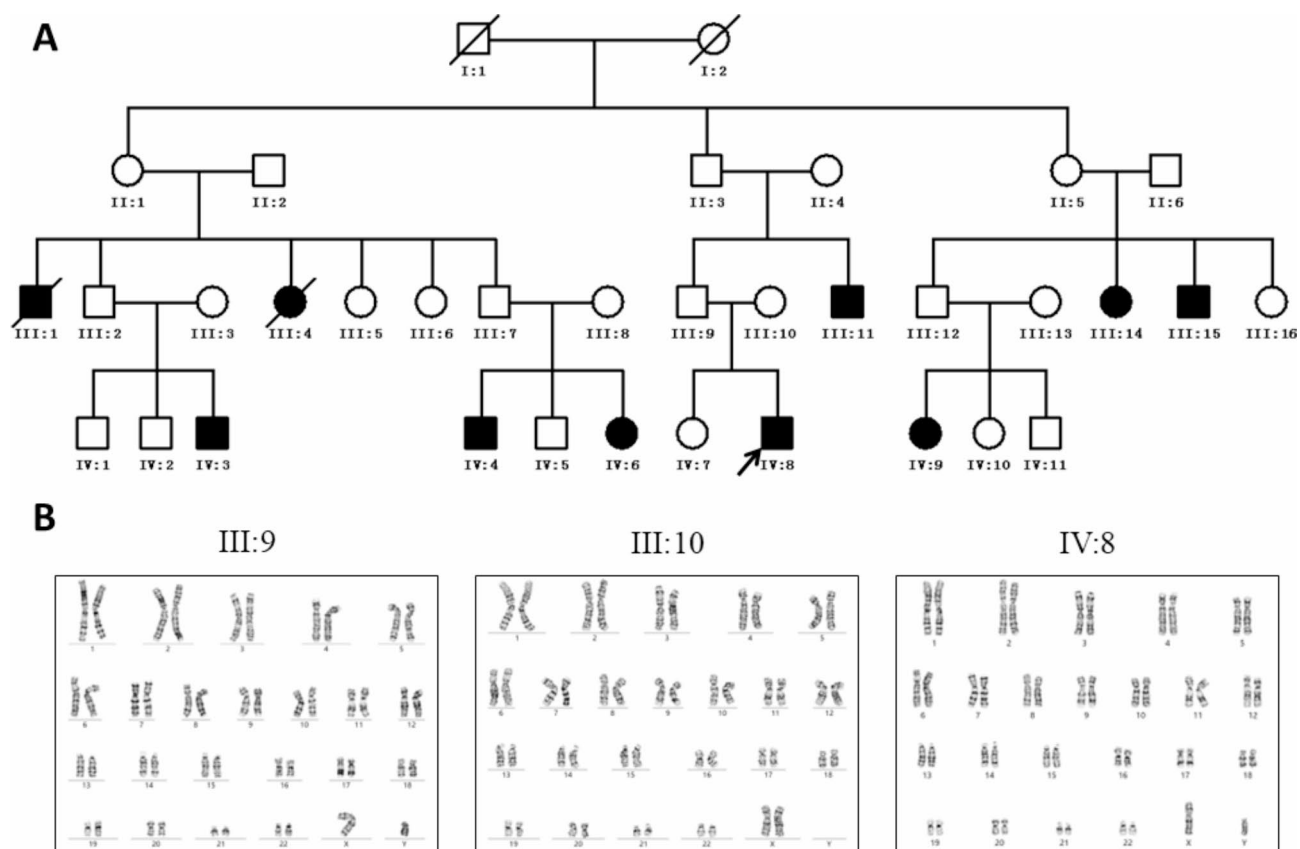


Fig. 1. Family pedigree, clinical sign and karyotype of the proband. (A) Family pedigree of the proband (IV:8, indicated by an arrow). (B) Karyotype of the proband (IV:8) and his parents (III:9, III:10) determined by G-banding analysis.

could not detect any pathogenic single nucleotide variants or indels related to the clinical phenotype of the patients in another genetic laboratory of a tertiary hospital.

Repeat test for *FMR1* and *NOTCH2NLC*.

The pedigree and clinical phenotypes suggest that the intellectual disability may be caused by a repeat expansion disease¹⁵. Therefore, we performed the RP-PCR testing for CGG repeats of *FMR1* and GGC repeats of *NOTCH2NLC* in the proband and his parents. The results showed that the CGG repeats of *FMR1* and GGC repeats of *NOTCH2NLC* were within the normal range (Fig. S1; Table S2).

Microdeletion and microduplication detected by Nanopore sequencing

Whole genome long-read nanopore sequencing analysis was performed on the proband to detect potential pathogenic variants. A total of 5,480,958 reads with 108,047,618,717 bases were obtained (~34 × read coverage of the haploid genome), with a mean length of 20,378 bases and a median length of 33,825 bases. More than 99% of reads were aligned to the human reference genome (GRCh37/hg19). The Sniffles2 SV calling identified a 3.26 Mb microduplication region on chr12 and a 1.2 Mb microdeletion region on chr22, with average sequencing depths on these regions of 50× and 16×, respectively.

The subject had heterozygous deletion mutations in the chr22:49736725–51,244,566 regions, including 48 genes, including *SHANK3*, *TUBGCP6*, and *ALG12*. (Fig. 2; Table 1). The Decipher and MedlinePlus databases show that this deletion is associated with the 22q13.3 deletion syndrome (PMS), in which *SHANK3* is a single dose deficient gene^{2,16–18}. Based on the above evidence and according to the ACMG guideline for copy number variants¹⁹, this repeated variant can be rated as a pathogenic variant. The duplication variation of the subject in the chr12:130583071–133,851,895 region (including 58 genes such as *POLE*, *P2RX2*, and *PUS1*) does not overlap with a clear triple dose sensitive gene or benign region (Fig. 2; Table 1). The patient's phenotype and similar repeated variation case phenotype reported in the Decipher database show that this repeated variation

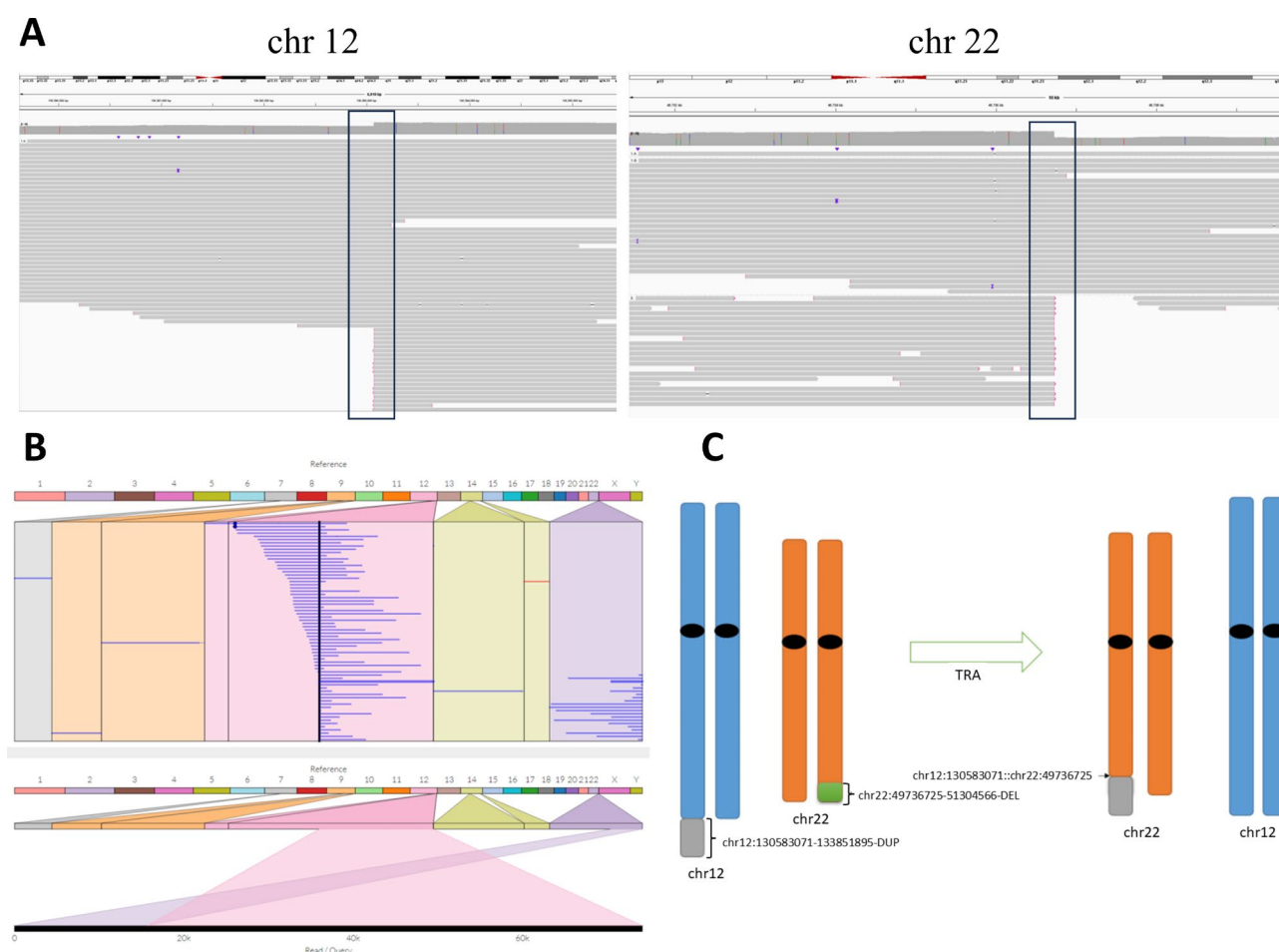


Fig. 2. Identification of chromosome breakpoints of the proband and visualized analysis of nanopore sequencing data. (A) Breakpoints of Chromosomes 12 and 22 detected by long-read nanopore sequencing are shown in IGV and the area in the box is the breakpoint reads. DNA fragments were compared to the reference human genome (GRCh37/hg19). (B) Visualization of the breakpoints in target chromosomes with ribbon. The breakpoint of chromosome 12 is connected to the breakpoint of chromosome 22. (C) Schematic diagram of chromosome structure variation according to nanopore sequencing results.

NO.	Chromosome location	break location	Variation interval	size	type	Population frequency	Dose sensitive genes	Pathogenicity
1	12q24.33	Chr12:130583071	Chr12: 130,583,071–133,851,895	3.26 Mb	DUP	0	none	VUS
2	22q13.33	Chr22:49736725	chr22:49736725–51,304,566	1.5 Mb	DEL	0	SHANK3	Pathogenic

Table 1. Structure variants detected by Nanopore sequencing. DUP: duplication; DEL: deletion; VUS: variants of uncertain significance.

can be rated as VUS according to the ACMG guideline¹⁹. Additionally, the precise breakpoints are located at chr12:130583071 and chr22:49736725 retrospectively, and the breakpoint positions do not involve genes (Fig. 2B).

Breakpoint validation by PCR sequencing

To further validate the exact translocation breakpoints of the proband and his family members, PCR amplification was performed. Meanwhile, Sanger sequencing was performed for the proband’s father (III:9) at the level of single bases. We detected and validated the breakpoints of chr12:130583071 and chr22:49736725 (Fig. 3A and B, Fig. S2). More importantly, PCR and nanopore sequencing results showed that the exact karyotype of the proband’s father (III:9) was 46,XY, t(12;22) (q24.33;q13.33), and those of the other family members are show in Table 2, which are in accordance with their clinical characteristics (Fig. 1A).

Haplotype detection

Haplotype identification of chromosomes is important for preimplantation genetic testing for structural rearrangement (PGT-SR); such adjacent SNP information can be used to predict the presence or absence of balanced translocation at single cell level^{20,21}. In this study, we performed haplotype analysis by the precise location of the breakpoints in the proband’s father (III:9). We found informative SNPs near the breakpoint regions (Fig. 3C), which enabled differentiation of the chromosomal region involved in translocation. The informative SNPs near the breakpoint within 2 Mb are listed in Table S3, and the 12 informative SNPs near the breakpoint are shown in Fig. 3C.

Discussion

To the best of our knowledge, this is the first study to directly diagnose PMS by nanopore sequencing in a four-generation Chinese family with cryptic unbalanced translocations. Our findings suggest that nanopore sequencing can serve as a powerful tool for the molecular diagnosis of suspected PMS patients and haplotype identification of chromosomal rearrangements in PGT-SR treatments.

PMS is a rare genetic disease caused by haploinsufficiency of SHANK3 due to 22q13.3 deletion or SHANK3 mutation^{2,3}. Nearly 90% of PMS are caused by the deletion of 22q13.3 including SHANK3 (Nevado), and the deletion size can vary from < 50 kb to 9.2Mb^{8,22}. SHANK3 is associated with disease via haploinsufficiency, which encodes a large scaffolding protein as severe as a scaffold to organize excitatory postsynaptic densities through protein-protein interactions^{23,24}. Thus, SHANK3 plays a critical role in glutamate transmission, synaptic spine dynamics, and learning and memory processes. Therefore, the major neurodevelopmental features of PMS, including global developmental delay and intellectual disability, are caused by the defects in SHANK3²⁵. In this study, a pathogenic heterozygous microdeletion of 1.5 Mb, including SHANK3 at chromosome 22q13.33 was identified by nanopore sequencing the proband (Fig. 2, IV:8). The clinical features of the proband, including profound intellectual disability, severe speech delay, behavioral abnormalities, and ASD, are highly consistent with those of the previous studies^{8,22,25}.

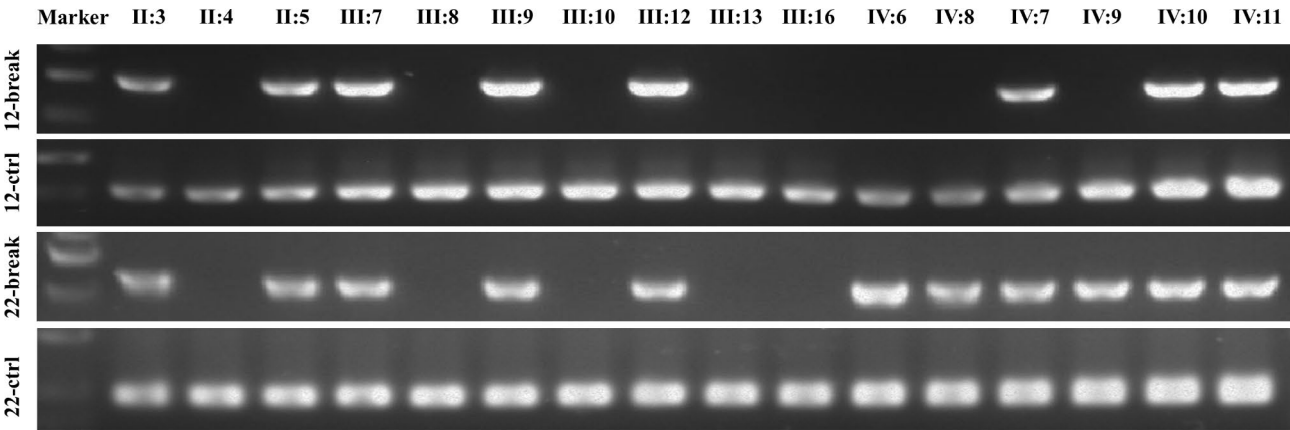
Given the significant heterogeneity in the severity and breadth of the clinical characteristics^{5,18,22}, it is difficult to choose a molecular testing method specific to PMS patients. According to the core features of PMS, differential diagnosis of Angelman syndrome, Prader-Willi syndrome, Fragile X syndrome, FG syndrome type 1, and Sotos syndrome is crucial for management^{6,26}. Furthermore, SHANK3 has been poorly covered by WES because of chromosomal terminal location and high GC content. Therefore, nanopore sequencing can play a unique and important role in the diagnosis of PMS and even rarer diseases, which is of great value for shortening diagnosis time and reducing unnecessary costs.

Relative to NGS, the read length of nanopore sequencing was much longer (with an average read length exceeding 10 kb), which can compensate for many shortcomings of NGS^{27,28}. Owing to its high sensitivity in detecting SVs and genome coverage, nanopore sequencing can detect complex types of structural variations and accelerate the disease-causing variants, such as repetitive regions and large genomic rearrangements²⁹. In this study, conventional genetic testing methods, including karyotyping, repeat tests of FMR1 and NOTCH2NCL, and trio-WES testing, failed to detect the cause of the severe intellectual disability of the proband in a large family. However, nanopore sequencing rapidly detected a pathogenic variant of 22q13.33 deletion with 1.5 Mb of the proband, which suggests that it can serve as a powerful tool for the diagnosis of rare genetic diseases.

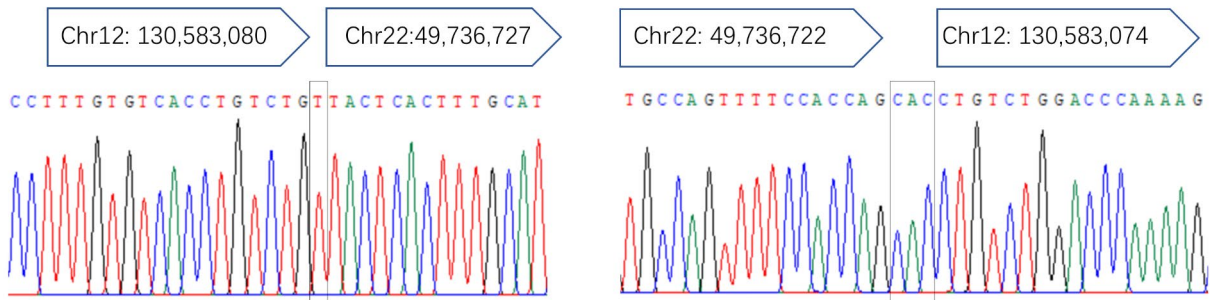
Currently, the prevalence and incidence of PMS are unknown and likely to be underestimated^{3,30}. With the wider application of genome sequencing technology, we will have a deeper and more comprehensive understanding of PMS. Additionally, solid evidence for the clinical efficacy of nanopore sequencing in the diagnosis of PMS and even rarer diseases still requires large-scale prospective cohort studies for confirmation.

In conclusion, this study described the application of nanopore sequencing in identifying a pathogenic variant of 22q13.33 microdeletion (1.2 Mb) of an 11-year-old child with severe intellectual disability in a four-generation Chinese family. The precise location of chromosomal breakpoints and the accurate karyotypes of

A



B



C

SNP				SNP			
F1		F0		F1		F0	
chr12	116646451	G	A	chr22	29757090	C	T
chr12	117925801	C	G	chr22	31622457	G	A
chr12	119294323	A	T	chr22	33446483	A	C
chr12	120537273	T	G	chr22	35261397	G	A
chr12	121770424	C	T	chr22	37025274	C	A
chr12	123013682	C	T	chr22	38802512	G	A
chr12	124213454	C	G	chr22	40596624	A	T
chr12	125586605	T	C	chr22	42402386	G	A
chr12	126721588	G	A	chr22	44287629	G	A
chr12	128000892	T	C	chr22	46071326	T	C
chr12	128975836	T	C	chr22	48100209	G	A
chr12	130581802	C	T	chr22	49736163	A	T
chr12	130583071	C	C	chr22	49736725	C	C
chr12	130583080	G	G	chr22	49736727	T	T
chr12	130592475	T	C	chr22	49739521	G	A
chr12	130895222	T	C	chr22	49882975	C	T
chr12	131217685	T	C	chr22	50103809	C	A
chr12	131513119	T	C	chr22	50253820	G	A
chr12	131824017	G	A	chr22	50414983	T	C
chr12	132077820	A	C	chr22	50557120	A	C
chr12	132331936	T	C	chr22	50706546	C	A
chr12	132597669	A	G	chr22	50853811	T	G
chr12	132909750	T	C	chr22	50993420	G	A
chr12	133264904	A	G	chr22	51081332	C	T
chr12	133501738	C	T	chr22	51171693	G	A
chr12	133839682	C	G	chr22	51242271	G	C

Fig. 3. PCR analysis, Sanger sequencing, and haplotype analysis for the breakpoints according to the reference human genome (GRCh37/hg19). (A)PCR analysis for validating the breakpoints of the family members. (B) Sanger sequencing for the breakpoints of chromosomes 12 and 22 of the proband’s father (III:9). (C) Haplotype analysis for preimplantation genetic testing for structural rearrangement according to the nanopore sequencing of III:9. Yellow blocks represent male’s informative SNPs, F0 represents the normal chromosome, and F1 represents the translocation chromosome. Light purple and light green represent the male’s two translocation chromosomes retrospectively.

the proband and other family members were determined by PCR and Sanger sequencing, which is helpful for providing effective genetic counseling and reproductive strategy guidance. Additionally, the haplotype was also constructed by nanopore sequencing, which can help the family prevent the risk of transmission of this cryptic balanced translocation through PGT-SR technology. We believe that the nanopore sequencing can play

Number of family member	PCR results of chr12-break	PCR results of chr-22-break	karyotype
II:3	+	+	46,XY, t(12;22)(q24.33;q13.33)
II:4	-	-	46,XX
II:5	+	+	46,XX, t(12;22)(q24.33;q13.33)
III:7	+	+	46,XY, t(12;22)(q24.33;q13.33)
III:8	-	-	46,XX
III:9	+	+	46,XY, t(12;22)(q24.33;q13.33)
III:10	-	-	46,XX
III:12	+	+	46,XY, t(12;22)(q24.33;q13.33)
III:13	-	-	46,XX
III:16	-	-	46,XX
IV:6	-	+	46,XX, dup(12)(q24.33); del(22)(q13.33)
IV:8	-	+	46,XY, dup(12)(q24.33); del(22)(q13.33)
IV:7	+	+	46,XX, t(12;22)(q24.33;q13.33)
IV:9	-	+	46,XX, dup(12)(q24.33); del(22)(q13.33)
IV:10	+	+	46,XX, t(12;22)(q24.33;q13.33)
IV:11	+	+	46,XY, t(12;22)(q24.33;q13.33)

Table 2. Precision karyotype of the family members according to PCR results of chr12-break and chr22-break. PCR: polymerase chain reaction; WT: wild type; t: translocation; dup: duplication; del: deletion; +: positive; -: negative.

an important role in the genetic diagnosis of PMS and rare diseases, chromosomal translocation detection, and PGT treatment in the near future.

Data availability

The sequence data generated in the current study have been deposited in the NCBI database with accession ID of PRJNA1200925.

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Author contributions

JC designed the experiments. QX and GC performed the experiments. JC and XW analyzed the experimental results and data. XW wrote the manuscript. JC, YZ and JLH supervised the study and offered suggestions for the revision of the manuscript. All authors participated in the discussion and approved the final manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Ethics statement

The study related to human participants was reviewed and approved by the reproductive medicine ethics committee of Jiangxi maternal and child health hospital. All patients participated in this study were signed written informed consent. The research has been performed in accordance with the Helsinki Declaration.

Additional information

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