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Carrier screening for multiple complex monogenic diseases using long-read sequencing: a population-based study of premarital couples in Shanghai

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Abstract

Background Carrier screening for severe recessive genetic diseases in couples undergoing premarital examinations is a crucial strategy for reducing the incidence of birth defects and promoting reproductive health. However, many high-prevalence but genetically complex diseases cannot be reliably detected using conventional PCR-based methods or short-read next-generation sequencing (NGS).

Results In this study, 1,203 couples who received free premarital medical examinations at seven units in Shanghai were recruited. PacBio long-read sequencing (LRS) was applied for simultaneous carrier screening of five genetically complex monogenic diseases, including spinal muscular atrophy (SMA), α-/β-thalassemia, congenital adrenal hyperplasia (CAH) (refers to 21-hydroxylase deficiency, 21-OHD), and fragile X syndrome (FXS). A total of 161 individuals were identified as carriers of a single disease, while four individuals carried pathogenic variants associated with two distinct diseases. Four couples were determined to be at high reproductive risk, including one classic CAH family, one SMA family, one hemoglobin H (Hb H) disease family, and one family in which the female was an FXS premutation carrier. In addition, one couple at risk of having a child with non-classic CAH (NCCAH), as well as one male individual with a confirmed diagnosis of NCCAH, were identified.

Conclusions LRS provides substantial clinical value for comprehensive carrier screening in the premarital population. It enables accurate detection of structural variants and repeat expansions that are often missed by conventional

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methods. These findings support the integration of LRS into routine premarital genetic screening protocols to enhance early identification of at-risk couples and improve reproductive decision-making.

Keywords Long-read sequencing (LRS), Premarital examinations, Premarital carrier screening, Spinal muscular atrophy (SMA), Thalassemia, congenital adrenal hyperplasia(CAH), Fragile x syndrome (FXS)

Background

Birth defects represent a major global public health concern and are among the leading causes of infant mortality and long-term disability. Globally, an estimated eight million newborns are affected by birth defects annually, accounting for about 6% of all births and resulting in nearly 500,000 neonatal deaths each year [1]. In China, the estimated prevalence of birth defects is 5.6%, with around 900,000 new cases reported each year [2]. Birth defects not only compromise child survival and quality of life but also impose substantial socioeconomic burdens, including shortened life expectancy, increased healthcare expenditures, and psychosocial stress on families. Moreover, the high incidence of birth defects adversely impacts population health indicators, posing a major challenge for reducing infant mortality and improving national life expectancy metrics.

Premarital medical examination (PME) refers to a systematic health assessment conducted before marriage registration, aiming to identify infectious diseases, genetic disorders, and reproductive system abnormalities that may affect spousal health or offspring risks [3]. PME serves as a critical preventive strategy for improving birth outcomes and promoting reproductive health. It offers unique advantages, such as access to a concentrated, reproductive-age population, opportunities for early intervention, and manageable costs. PME represents an ideal entry point for genetic disease screening and counseling, particularly among asymptomatic individuals with no apparent family history. The identification of carriers of recessive pathogenic variants at the preconception stage enables informed reproductive decision-making, supports primary prevention of inherited disorders, and maximizes the public health value of premarital care services [4].

Current approaches for carrier screening of recessive pathogenic variants include pedigree analysis, targeted variant detection assays, and next-generation sequencing (NGS)-based approaches such as gene panels, whole-exome sequencing (WES), and whole-genome sequencing (WGS) [5–10]. Pedigree analysis, although cost-effective, relies heavily on an accurate family history and cannot detect de novo variants [5]. Targeted variant detection assays, such as quantitative polymerase chain reaction (qPCR) and amplification refractory mutation system-polymerase chain reaction (ARMS-PCR), offer high sensitivity for known variants but are limited in scope and unable to detect rare or novel variants [6, 7]. NGS enables broad detection of single-nucleotide variants (SNVs), small insertions and deletions (indels), and structural variants and has become a cornerstone technology in carrier screening due to reduced costs and improved accessibility [8–10]. However, several monogenic disorders with high carrier frequencies remain challenging to detect accurately with short-read NGS due to complex genomic features, such as extremely long genes, high homology with pseudogenes, and large structural rearrangements. For example, spinal muscular atrophy (SMA) is primarily caused by loss or variants of *SMN1* (~ 28 kb), which has a highly homologous modifier gene *SMN2*, making it difficult to distinguish between the two genes [11]. α-/β-thalassemia involves large deletions or complex recombination events in *HBA1*, *HBA2*, and *HBB*, which are often unresolved by standard NGS [12]. Congenital adrenal hyperplasia (CAH), predominantly resulting from *CYP21A2* defects (21-hydroxylase deficiency, 21-OHD), is complicated by 99.8% sequence identity shared with the pseudogene *CYP21A1P*, leading to frequent misalignment and inaccurate variant detection [13]. Fragile X syndrome (FXS) results from CGG repeat expansions at the *FMR1* gene, which may exceed 1,000 repeats and frequently involve complex structural patterns such as AGG interruptions, requiring specialized commercial assays [14]. For FXS diagnosis, specialized PCR-based assays such as the AmplideX® FMR1 PCR/CE Reagent Kit (Asuragen) and the Molecular Fragile X PCR Kit (Abbott) are required [15]. Therefore, relying solely on conventional NGS for carrier screening of these complex, high-prevalence, technically challenging diseases may lead to missed or inaccurate diagnoses, ultimately reducing the effectiveness of genetic counseling and birth defect prevention efforts.

Third-generation sequencing technologies, especially long-read sequencing (LRS) platforms like PacBio, offer distinct advantages for resolving these technically challenging genomic regions. LRS produces continuous long reads, avoids PCR amplification bias, and achieves high base-level accuracy (>99.9%) [16]. These features allow for precise characterization of structural variants, complex recombination events, and repeat expansions. LRS enables accurate quantification and differentiation of *SMN1* and *SMN2*, reliable detection of diverse variant types in *HBA1*, *HBA2*, and *HBB*, precise recombination analysis of *CYP21A2* and its highly homologous pseudogene *CYP21A1P*, as well as high-resolution sizing and characterization of CGG repeat expansions in *FMR1* [11,

12, 14, 17, 18]. Furthermore, LRS supports simultaneous detection of multiple disorders in a single sequencing run, offering high throughput, high sensitivity, and the potential for streamlined workflow integration. A recent retrospective study has confirmed the superior diagnostic yield of LRS over short-read NGS in identifying pathogenic variants in complex genomic regions [18]. Integrating LRS into current NGS-based carrier screening workflows may significantly improve carrier detection sensitivity and risk stratification in premarital populations, advancing precision reproductive medicine and contributing to national birth defect prevention initiatives.

In this study, we applied PacBio LRS to a cohort of 1,203 couples undergoing PME in Shanghai, conducting concurrent carrier screening for five high-prevalence and technically challenging monogenic diseases: SMA, α-/β-thalassemia, 21-OHD, and FXS. This study aimed to evaluate the feasibility, technical advantages, clinical value, and public health implications of implementing LRS in the premarital examination population. The findings are expected to provide evidence supporting the adoption of advanced genomic technologies into national birth defect prevention strategies.

Methods

Participants

From March to July 2024, a total of 1,203 couples of reproductive age (2,406 individuals) undergoing free premarital medical examinations in Shanghai were recruited for this study. Participants were enrolled at the International Peace Maternity and Child Health Hospital and seven premarital medical examination institutions located in Minxing, Songjiang, Jinshan, Fengxian, Xuhui, and Pudong districts. Inclusion criteria were as follows: (1) both partners voluntarily completed a premarital medical examination at the participating institutions; (2) both partners provided written informed consent. Individuals were included only when both criteria were met. Exclusion criteria were: (1) individuals with infectious diseases; (2) samples unsuitable for testing due to improper collection, transportation, or storage; (3) mislabeled or otherwise confused samples; and (4) any other condition deemedunsuitable by investigators. All participants were clinically asymptomatic at enrollment. No pre-screening or exclusion based on personal or family history of hereditary diseases was performed, ensuring that the cohort represented a general low-risk population for carrier screening. All samples were collected in Shanghai. To assess regional genetic diversity within the screened population, the birthplaces or registered permanent residences of the participants were inferred from their national ID information. These data were used exclusively for demographic stratification and population genetic background analysis, and do not represent the physical locations of sample collection. This study was approved by the Ethics Committee of the International Peace Maternity and Child Health Hospital. Written informed consent was obtained from all participants prior to sample enrollment.

LRS analysis

Peripheral blood (2 mL) was collected from each participant using EDTA anticoagulant tubes. Genomic DNA was extracted using the GenMagBio Genomic DNA Purification kit (GenMagBio, Changzhou, China). DNA concentration was measured using the Qubit dsDNA HS kit (Thermo Fisher Scientific, USA). Samples were considered qualified for sequencing if they met the following criteria: DNA concentration ≥ 50 ng/μL, A260/A280 ≈ 1.8, A260/A230 > 2.0, and no evidence of degradation on agarose gel electrophoresis.

LRS was performed by Berry Genomics Corporation (Beijing, China) following previously described protocols with minor optimization [18]. Full-length target regions associated with SMA (*SMN1*, *SMN2*), α-thalassemia (*HBA2*, *HBA1*), β-thalassemia (*HBB*), 21-OHD (*CYP21A2*, *CYP21A1P*, *TNXB*, *TNXA*) and FXS (*FMR1*) were amplified by multiplex long-range PCR. Specific experimental details, including primer sequences, amplicon lengths, and amplification conditions are provided in the supplementary materials. Amplicons were purified, end-repaired, and ligated with barcoded adapters. Unligated fragments were removed via exonuclease digestion. Sequencing libraries were prepared using the Sequel Binding and Internal Ctrl Kit 3.0 (Pacific Biosciences, Menlo Park, CA) and sequenced on the PacBio Sequel II platform (384 samples in one flow cell, 30-h runtime). High-fidelity Cyclic Consensus Sequencing (CCS) reads were generated using SMRT Link software. Barcode demultiplexing and adapter trimming were performed using Lima software. CCS reads were aligned to the GRCh38/hg38 human reference genome using pbmm2. Disease-specific bioinformatics pipelines were applied to detect pathogenic variants [18]. Variant classification followed the American College of Medical Genetics and Genomics (ACMG) guidelines, referencing ClinVar and the Human Gene Mutation Database (HGMD) [19]. CCS reads of representative samples were displayed in the Integrated Genomics Viewer (IGV) plots and Waterfall plots to demonstrate LRS test results. For quality control, target regions were completely detected three or more times were considered valid HiFi CCS reads. After barcode splitting, only HiFi CCS reads meeting the following minimum thresholds were included in variant interpretation: ≥200 for SMA (*SMN1*, *SMN2*) and 21-OHD (*CYP21A2*, *CYP21A1P*, *TNXB*, *TNXA*), ≥ 60 for α-thalassemia (*HBA2*, *HBA1*) and β-thalassemia (*HBB*), ≥

100 for the full mutation of *FMR1*, ≥ 2000 for the normal, intermediate, premutation, and mosaicism of *FMR1*, and ≥ 30 for the intragenic SNVs and indels of *FMR1*.

Calculation of carrier frequency

Carrier frequencies were calculated based on the presence of variants classified as pathogenic (P) or likely pathogenic (LP) according to ACMG/AMP guidelines. Variants of uncertain significance (VUS) were excluded from frequency analysis. Individuals harboring multiple P/LP variants in the same gene were counted only once to avoid overestimation. For thalassemia, individuals harboring copy number gains involving *HBA1*/*HBA2* were not classified as carriers because such genotypes typically do not manifest disease. However, these individuals were flagged as high-risk when their partners carried β-globin gene variants, due to the potential for producing offspring with "Type III thalassemia". The term "Type III thalassemia" refers to a condition in which an individual simultaneously carries an increased copy number of the α-globin gene and a variant in the β-globin gene. This condition exacerbates the imbalance between the α-globin and β-globin chains in patients, potentially leading to more severe anemia. For 21-OHD, only variants classified as salt-wasting (SW) or simple virilizing (SV) subtypes according to the European Molecular Genetics Quality Network (EMQN) were included. For SMA, carrier status was determined based on *SMN1* copy number only. *SMN2* copy number, while clinically relevant for predicting disease severity in affected fetuses, does not influence carrier classification and was therefore not included in the carrier frequency analysis. For the X-linked (XL) inherited disease FXS, only female carriers with premutation-range *FMR1* CGG repeats (55-200 repeats) were included in the carrier frequency calculation.

Table 1 Carrier frequencies of five hereditary disorders in the study cohort (*N* = 2,406)

Disease(s) carried in a person	Number of samples	Carrier frequency (%)
SMA	37	1.54%
α-Thalassemia	78	3.24%
β-Thalassemia	10	0.42%
21-OHD	35	1.45%
FXS*	1	0.08%
α-Thalassemia & β-Thalassemia	1	0.04%
α-Thalassemia & 21-OHD	1	0.04%
α-Thalassemia & SMA	1	0.04%
β-Thalassemia & SMA	1	0.04%
Total	165	-

*Only female individuals with premutation-sized *FMR1* CGG expansions (55-200 repeats) were considered carriers for FXS. The calculated carrier frequency was 0.08% (1/1203)

Carrier frequency for each condition was calculated as the number of individuals harboring P/LP variants (for SMA, defined by P/LP SNVs/indels or copy-number losses of *SMN1*; for FXS, defined by premutation-range CGG repeats in females; for thalassemia, defined by P/LP SNVs/indels or copy-number losses; for 21-OHD, defined by EMQN-classified SW/SV variants) divided by the total number of individuals successfully sequenced for that gene (2406 for SMA, thalassemia and 21-OHD; 1203 for FXS). No additional statistical modeling was applied.

Genetic counseling and follow-up

(1) For affected individuals, appropriate clinical interventions were recommended when necessary to support physiological health and reproductive planning. (2) For couples classified as high genetic risk based on carrier status, comprehensive genetic counseling was provided. Reproductive options were discussed, including the recommendation of prenatal diagnosis using LRS during natural pregnancy or preimplantation genetic testing for monogenic diseases (PGT-M), to prevent the birth of affected offspring.

Results

Overall carrier screening results

A total of 1,203 couples (2,406 individuals) were included in this study. The overall carrier frequency of at least one of the five screened monogenic diseases was 6.86% (165/2406). Among them, 161 individuals (6.69%) were carriers of a single disease, while four individuals (0.17%) carried P/LP variants for two different diseases (Table 1). The diseases ranked by carrier frequency were α-thalassemia (81/2406, 3.37%), 21-OHD (36/2406, 1.50%), SMA (39/2406, 1.62%), β-thalassemia (12/2406, 0.50%), and FXS (1/1203, 0.08%).

In total, 39 P/LP variants were detected, including three *SMN1* variants, 12 α-thalassemia variants, eight β-thalassemia variants, 15 *CYP21A2* variants, and one *FMR1* premutation (Table 2). Among these, 18 variants (46.15%) were detected only once. The most frequently detected variant was Δ7SMN1, which was detected in 37 individuals. Other recurrent variants included $-\alpha^{3.7}$ (α-thalassemia, $n = 24$), *HBA2*: c.301-24delinsCTCGGCCC (α-thalassemia, $n = 15$), $--^{SEA}$ (α-thalassemia, $n = 14$), *CYP21A2*: c.293-13C>G (21-OHD, $n = 10$). In addition, LRS identified increased α-globin gene copy number in several individuals, including 47 cases of α-triplication (1.95%), three cases of α-tetraploidy (0.12%), and 10 cases of HKαα (0.42%) (Table 3). No β-thalassemia variants were detected among the spouses of these individuals. Therefore, no couples were classified as being at risk of producing offspring with "Type III thalassemia".

Table 2 Pathogenic variants and corresponding carriers detected in the cohort (*N* = 2,406)

Disease	Gene	Variant type	Variant	Carriers of the variant	Frequency (%)	Carriers of the gene	Frequency (%)
SMA	*SMN1*	Deletion	Δ7*SMN1*	37	1.54%	39	1.62%
		SNV/Indel	c.22dup	1	0.04%		
			c.30del	1	0.04%		
α-Thalassemia	*HBA1*, *HBA2*	Deletion-α^0	$--^{SEA}$	14	0.58%	81	3.37%
		Deletion-α^+	$-a^{3.7}$	24	1.00%		
			$-a^{4.2}$	7	0.29%		
		SNV/Indel	*HBA2*: c.-22C>T	2	0.08%		
			HBA2: c.46G>A	5	0.21%		
			HBA2: c.301-24delinsCTCGGCCC	15	0.62%		
			HBA2: c.369C>G	2	0.08%		
			HBA2: c.*47G>C	1	0.04%		
			HBA1: c.84G>T	1	0.04%		
			HBA1: c.95+9C>T	3	0.12%		
			HBA1: c.301 − 31_301-24delinsG	2	0.08%		
			HBA1: c.*134_*135insT	5	0.21%		
β-Thalassemia	*HBB*	SNV/Indel	*HBB*: -78A>G	1	0.04%	12	0.50%
			HBB: c.52A>T	2	0.08%		
			HBB: c.79G>A	1	0.04%		
			HBB: c.84_85insC	2	0.08%		
			HBB: c.93-3T>G	1	0.04%		
			HBB: c.126_129delCTTT	2	0.08%		
			HBB: c.315+65T>C	1	0.04%		
			HBB: c.316-179A>C	2	0.08%		
21-OHD	*CYP21A2*	30-kb Deletion-SW	*CYP21A1P/CYP21A2*_CH-1	5	0.21%	36	1.50%
			*CYP21A1P/CYP21A2*_CH-3	2	0.08%		
			*TNXA/TNXB*_CH-1	2	0.08%		
			*TNXA/TNXB*_CH-3	3	0.12%		
		30-kb Deletion-SV	*CYP21A1P/CYP21A2*_CH-4	1	0.04%		
		SNV/Indel-SW	c.293-13C>G	10	0.42%		
			c.535G>A	1	0.04%		
			E6_cluster	1	0.04%		
			c.923dup	1	0.04%		
			c.955C>T	1	0.04%		
			c.955C >T & c.1069C >T	1	0.04%		
			c.1069C>T	1	0.04%		
			c.1451_1452delinsC	1	0.04%		
		SNV/Indel-SV	c.518T>A	5	0.21%		
			c.1451G>C	1	0.04%		
FXS*	*FMR1*	Premutation	CGG 29/75/87 repeats	1	0.08%	1	0.08%

SNV, single-nucleotide variants, *Indel*, small insertions and deletions; α^0, two α-globin genes disrupted; α^+, one α-globin gene disrupted; SW, salt-wasting; SV, simple virilizing; NC, non-classical

*Only female individuals with premutation-sized *FMR1* CGG expansions (55-200 repeats) were considered carriers for FXS. The calculated carrier frequency was 0.08% (1/1203)

Table 3 Detected α-globin structural variants in the study cohort (*N* = 2,406)

Variant	Carriers of the variant	Frequency (%)
$\alpha\alpha^{anti3.7}$	29	1.21%
$\alpha\alpha^{anti4.2}$	18	0.75%
$\alpha\alpha\alpha^{anti3.7}$	1	0.04%
$\alpha\alpha\alpha^{anti4.2}$	2	0.08%
HKαα	10	0.42%

Identification of high-risk couples

Couples were classified as high risk for genetic reproductive outcomes if both partners carried P/LP variants in the same autosomal gene, or if the female partner carried an X-linked P/LP variant. Based on these criteria, four couples were identified as being at risk of transmitting monogenic diseases to their offspring (Table 4). In one couple, both partners carried a single functional copy of

Table 4 At-risk couples identified for the five Monogenic disorders included in this study

Family number	Disease	Gene	Inheritance pattern	Genotype (Male)	Genotype (Female)	Comment
FAM01	SMA	*SMN1*	AR	[1 + 0]	[1 + 0]	The offspring will have a theoretical 25% chance of having SMA.
FAM02	21-OHD	*CYP21A2*	AR	c.293-13C>G/WT	c.293-13C>G/WT	The offspring will have a theoretical 25% chance of having classic CAH.
FAM03	α-Thalassemia	*HBA1*, *HBA2*	AR	$-\alpha^{3.7}$/WT	$--^{SEA}$/WT	The offspring will have a theoretical 25% chance of having Hb H disease.
FAM04	FXS	*FMR1*	XL	CGG 29 repeats	CGG 29/85/97 repeats	A risk of FXS in the offspring (especially boys)

WT wild type

SMN1 ([1 + 0]), conferring a 25% risk of SMA in offspring due to biallelic *SMN1* deletion (Fig. 1A). One couple was heterozygous for the same *CYP21A2* classic SW variant, c.293-13C>G, which carries a 25% risk of offspring with classic CAH (Fig. 1B). In the third family, the husband carried the α^+-thalassemia deletion $-\alpha^{3.7}$, and the wife carried the α^0-thalassemia deletion $--^{SEA}$. This combination confers a 25% risk of Hb H disease in offspring (Fig. 1C). In the fourth family, the female partner was found to carry two *FMR1* premutation alleles with 85 and 97 CGG repeats, placing male offspring at elevated risk of FXS (Fig. 1D). No couples were simultaneously at risk for more than one of the five screened diseases.

Genetic counseling and follow-up

All four families did not plan to conceive immediately but expressed clear intentions for future pregnancy. The SMA and classic CAH couples (FAM01 and FAM02) elected to pursue PGT-M for future pregnancies. The Hb H disease couple (FAM03) and the FXS family (FAM04), in which male offspring are at increased risk, chose to conceive naturally and undergo prenatal diagnosis during pregnancy. In addition to these high-risk families, one NCCAH high-risk couple and one male patient with NCCAH were also identified. The NCCAH high-risk couple carried different non-classic CYP21A2 variants (c.844G>T in the husband and c.143A>G in the wife), resulting in a 25% theoretical risk of NCCAH in their offspring (Fig. 1E). Given that NCCAH is typically associated with mild clinical manifestations and good prognosis, routine prenatal diagnosis is not mandatory. Therefore, we provided genetic counseling to the couple to explain the inheritance pattern and clinical spectrum of NCCAH. Couples were encouraged to make informed reproductive decisions based on balanced medical information and personal values. Ultimately, this couple plans to conceive naturally and conduct newborn screening (17-OHP test) for the baby after birth. If there were any abnormalities, further genetic diagnosis would be made. For the NCCAH patient, he carried pathogenic compound heterozygous variants in the *CYP21A2* gene (c.518T>A/c.844G>T) (Fig. 1F). He was asymptomatic and was referred to endocrine specialists for follow-up. His partner had a wild-type genotype, so the probability of giving birth to an affected child is minimal. However, their children will be carriers, and this information should be retained for future reproductive counseling.

Discussion

This study is the first to systematically apply LRS for simultaneous carrier screening of five common but technically challenging monogenic diseases, namely SMA, α-/β-thalassemia, 21-OHD, and FXS, in a real-world cohort of couples undergoing free premarital medical examinations in Shanghai. We determined both the individual carrier rates for each condition and the frequency of compound carriers. Overall, 6.69% (161/2,406) of individuals were carriers of a single disease, whereas 0.17% (4/2,406) carried pathogenic variants in two different genes.

Although both LRS and conventional NGS can detect variants associated with SMA, α-thalassemia, and β-thalassemia (with NGS showing lower sensitivity), only LRS enables effective simultaneous screening for 21-OHD and FXS. The current diagnostic process for 21-OHD relies on multiplex ligation-dependent probe amplification (MLPA) combined with Sanger sequencing, which is a labor-intensive workflow unsuitable for large-scale screening [20]. Similarly, FXS testing typically depends on PCR-capillary electrophoresis-based assays, which lack scalability and multiplexing capability [21]. Compared with conventional PCR-based and NGS approaches, LRS offers several advantages. First, it enables the simultaneous detection of diverse variant types, including deletions, recombinations, and repeat expansions, thereby enhancing detection accuracy. Second, the long read length facilitates the precise resolution of highly homologous sequences and pseudogenes, which often confound short-read sequencing. Third, the direct sequencing of CGG repeat regions in *FMR1* allows for more accurate characterization without reliance on indirect primer-based amplification. Recent studies have provided additional evidence supporting these advantages, particularly in carrier screening and in resolving complex

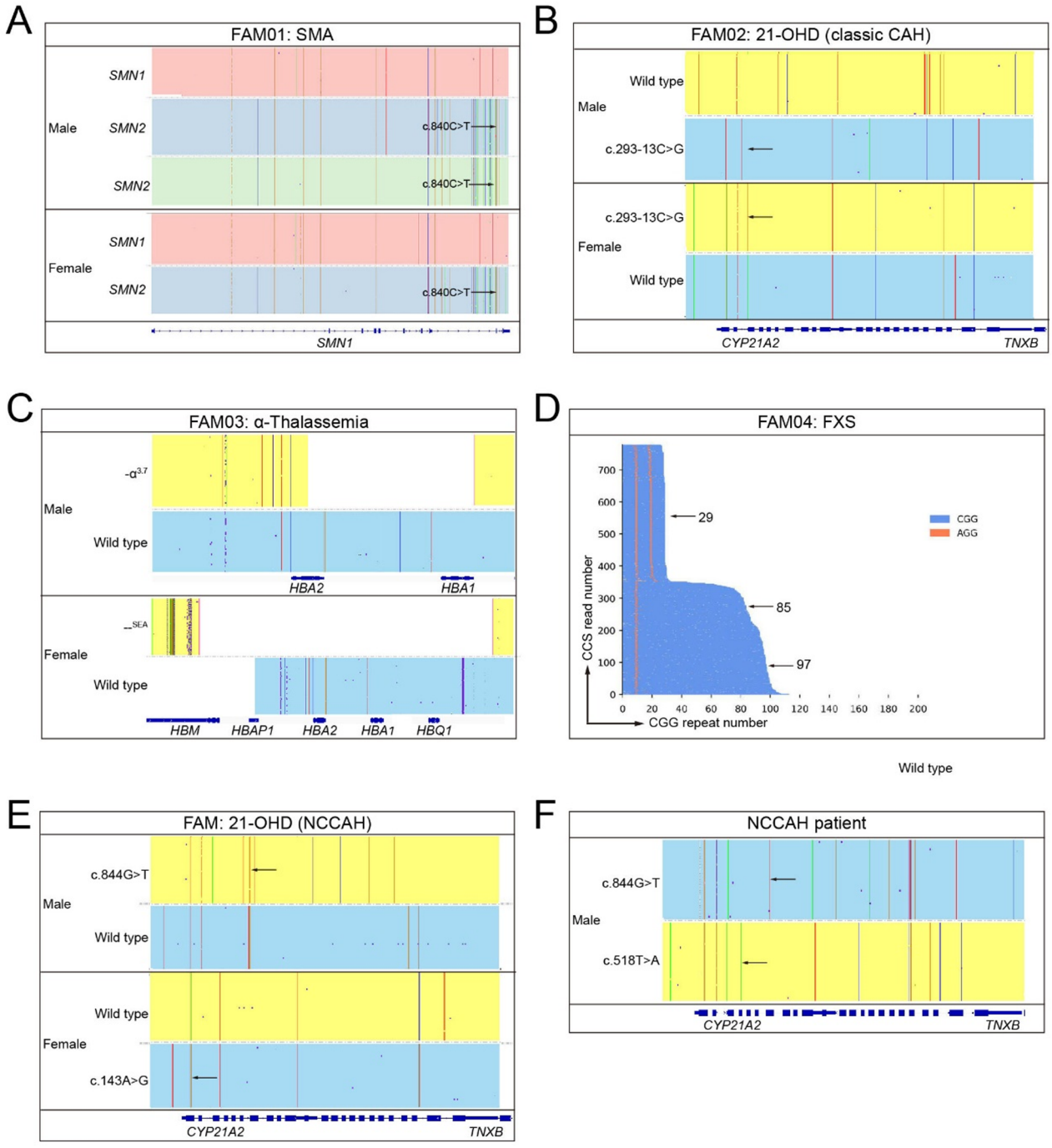

Fig. 1 Integrative genomics viewer (IGV) plots and waterfall plots of representative samples detected by LRS. **A**-**C** IGV plots displaying the at-risk couples identified for SMA (FAM01), classic CAH (FAM02), and α-thalassemia (FAM03). **D** Waterfall plots showing *FMR1* CGG repeats and AGG interruptions of members in family FAM04. **E** IGV plots displaying the at-risk couples identified for NCCAH. **F** IGV plots displaying the NCCAH patient carrying pathogenic compound heterozygous variants in the *CYP21A2* gene (c.518T>A/c.844G>T)

loci that are difficult to detect by short-read sequencing. For *SMN1* and *SMN2*, several LRS-based analyses have demonstrated improved accuracy in distinguishing homologous sequences and hybrid alleles, outperforming MLPA- or NGS-based methods in resolving copy number and gene conversion events [11, 22–24]. Similarly, LRS enables precise haplotype-level detection of α-/β-thalassemia deletions and recombinations, providing higher concordance with clinical phenotypes compared to conventional gap-PCR or combined assay systems [12, 25–30]. In *CYP21A2*, LRS offers a significant advantage in identifying rearrangements and pseudogene-derived

chimeric alleles that often escape capture by targeted NGS or MLPA [13, 20, 31, 32]. Moreover, direct measurement of the CGG repeat length in *FMR1* using LRS eliminates the need for primer-based enrichment and improves the classification of intermediate and premutation range expansions [14, 15, 33, 34]. Collectively, these findings support the technical advantages observed in our cohort and highlight the potential of LRS as an integrated platform for carrier screening of structurally complex genomic disorders. Notably, unlike previous studies that focused on a single gene or disease category, our work applies LRS to simultaneously screen five clinically significant and technically complex monogenic disorders in a real-world premarital population, thereby demonstrating its feasibility and scalability for public health–oriented carrier screening [18].

Beyond analytical accuracy, the practical applicability of LRS in population-based carrier screening also depends on its cost-effectiveness. In the present study, four disease-specific long-range amplification and sequencing workflows (SMA, thalassemia, 21-OHD, and FXS) were performed individually, resulting in an average per-individual cost of approximately 80 USD (including long-range PCR, library construction, and sequencing) [14, 17, 18, 22, 25]. The testing cost for each workflow is approximately 20 USD, which is comparable to or less than the combined cost of conventional methods that require screening for multiple diseases simultaneously, such as MLPA and qPCR for *SMN1* copy number detection [23, 24], gap-polymerase chain reaction (gap-PCR), reverse dot blot hybridization (RDB), MLPA and Sanger sequencing for thalassemia genotyping [26–30], MLPA and Sanger sequencing for *CYP21A2* complex variants [31, 32], and Southern blot analysis (SBA), triple primer PCR (TP-PCR) combined with capillary electrophoresis (CE) for *FMR1* repetitive sequence amplification [33, 34]. Multiplex long-range amplification and pooled barcoding pipelines are progressively enabling simultaneous interrogation of multiple genes associated with recessive diseases in a single workflow, substantially reducing reagent consumption and labor costs. Additionally, LRS eliminates the need for repeated confirmatory assays for complex structural variants, hybrid alleles, or unstable repeat expansions, which may further reduce total spending in clinical practice [14, 17, 22, 25]. Considering turnaround time, our approach required approximately 8 working days from DNA extraction to variant reporting, which is comparable to or faster than combined conventional workflows that often require sequential testing [18]. It should be noted that this turnaround time was achieved under a batch-processing workflow. In prospective settings, sample arrival patterns may introduce some variability in batching efficiency. However, in real-world LRS operations, sequencing runs are typically filled by consolidating samples from different hospitals, regions, or testing indications, allowing laboratories to reach full capacity rapidly. Such cross-project pooling substantially reduces waiting time and mitigates batching-related delays, resulting in an effective TAT that is close to the one observed in our study. Taken together, these findings suggest that while the current per-sample cost of LRS is still higher than that of single-disease conventional assays, it becomes economically competitive in real-world carrier screening programs involving multiple recessive conditions with complex genetic architectures. With further reduction in sequencing costs and integration of automated LRS pipelines, LRS has the potential to serve as a scalable and cost-effective platform for premarital, preconception, or newborn carrier screening within public health systems. Despite these strengths, an important limitation of this study is that the LRS findings were not confirmed by secondary methods. Although internal quality control and high sequencing depth were applied to ensure reliable variant detection, validation using an independent technique such as Sanger sequencing or MLPA would further increase confidence in the results. Future studies integrating orthogonal confirmation could help to reinforce the robustness of LRS-based population carrier screening.

We identified four high-risk reproductive couples: one with homozygous *SMN1* deletion (SMA), one with homozygous *CYP21A2* variant c.293-13C>G (classic CAH), one with a combination of $--^{SEA}$ and $-\alpha^{3.7}$ α-thalassemia deletions (Hb H disease), and one in which the female carried *FMR1* premutation alleles (85 and 97 CGG repeats), conferring increased FXS risk to male offspring. These findings underscore the importance of premarital or preconception carrier screening for early detection of autosomal recessive and X-linked disorders. To contextualize our findings, we compared the carrier frequencies observed in this Shanghai cohort with previous studies in Chinese and other East Asian populations. SMA carrier frequency (1.62%) aligns with reports from Qinzhou (1.64%) and Shenzhen area of China (1.64-1.70%) [11, 35]. For α-/β-thalassemia, carrier rates can differ significantly depending on the specific geographical region and ethnicity within China, with higher rates in southern regions that decline progressively northward (0.16-45.15% for α-thalassemia and 0.11-7.05% for β-thalassemia) [36]. Our cohort shows higher α-thalassemia (3.37%) and β-thalassemia frequencies (0.50%) than those reported in the nearby region Jiangsu province (2.62% for α-/thalassemia and 0.40% for β-thalassemia) [37]. PacBio HiFi sequencing has been demonstrated in multiple studies to generate long reads and accurately determine α-globin copy number variations (CNVs), showing near-perfect concordance with MLPA and gap-PCR results [38–40]. In this study, several internal quality control criteria to

ensure robustness of all variant detections, minimizing the risk of analytical errors even without MLPA/qPCR validation. Furthermore, reported frequencies of α-globin gene triplication in Chinese populations, including 0.77% in Guizhou province [41], 0.84% in southern Guangxi [42], 1.2% in Guangdong province [43], and 1.95% in Ganzhou city (southern China) [44], suggest that the incidence observed in our study cohort is biologically plausible. 21-OHD carrier frequency (1.50%) is similar with the prior report in Jiangsu province (1.51%) [45]. FXS premutation carriers in our cohort (0.08%) are consistent with that reported in Hong Kong (0.08%) [46], and slightly lower than that reported in Taiwan (0.13%) [47]. These data indicate that the frequencies observed in our study are broadly consistent with prior reports, with some regional variation. Given the high carrier frequencies and clinical impact of these five disorders, we recommend their routine inclusion in premarital/preconception screening panels to enhance the effectiveness of fertility risk assessment and birth defect prevention. It is important to note that although *SMN2* copy number does not influence SMA carrier classification and was therefore not included in the carrier frequency calculations, its clinical relevance becomes critical during reproductive counseling. For at-risk couples identified through carrier screening, *SMN2* assessment is essential in prenatal diagnosis because *SMN2* dosage acts as a major modifier of phenotypic severity [48]. Incorporating *SMN2* analysis into future large-scale screening studies may therefore provide a more comprehensive framework that links carrier detection with prenatal risk stratification and improved clinical decision-making.

For couples who both carry non-classic *CYP21A2* variants, or a combination of classical and non-classical variants, the theoretical risk of having a child with NCCAH is 25% [49]. NCCAH typically manifests as a mild disorder with post-pubertal onset and favorable prognosis [49]. Therefore, the ethical appropriateness of offering prenatal or preimplantation genetic testing (PGT) for such non-severe phenotypes remains controversial [50, 51]. From a reproductive ethics perspective, the priority is to ensure that couples receive comprehensive, unbiased genetic counseling. This includes clearly explaining the autosomal recessive inheritance pattern, outlining the mild clinical spectrum and long-term prognosis, and discussing postnatal monitoring and management strategies [51]. Reproductive decisions should be left to the couple, who should be supported in making informed choices based on their values, cultural context, and risk tolerance [51, 52]. Regardless of whether a couple chooses prenatal diagnosis, their autonomy must be respected, and healthcare providers should avoid medical coercion or stigmatization of genetic risk [53]. Emphasizing that individuals with mild genetic disorders often enjoy a good quality of life helps to prevent genetic discrimination and supports a balanced ethical framework that values both medical prudence and reproductive freedom [51–53]. As genetic screening becomes increasingly common, the medical community must continue to balance scientific accuracy with ethical responsibility, especially when dealing with conditions of mild or variable penetrance [50–53].

Additionally, one male with undiagnosed NCCAH was identified, carrying compound heterozygous *CYP21A2* variants (c.518T>A/c.844G>T). His spouse was wild-type, thus eliminating reproductive risk for offspring with CAH, but all offspring are predicted to be carriers. This highlights the importance of identifying not only asymptomatic carriers but also undiagnosed individuals with mild or subclinical recessive disorders during premarital screening [54]. Because symptoms of NCCAH often emerge during adolescence, such individuals may be incorrectly classified as healthy, allowing pathogenic variants to silently propagate across generations [20]. Therefore, premarital and preconception screening strategies should be broadened to include mildly affected patients and to offer their offspring appropriate carrier testing, thereby achieving a continuum of prevention from the individual to the family level [54, 55]. Combining phenotypic assessment with molecular diagnostics can significantlyimprove the detection and management of such conditions, contributing meaningfully to public health goals related to reproductive risk mitigation [54, 55].

Although sampling occurred in Shanghai, the screened population exhibited diverse regional origins due to substantial internal migration. Individuals originated from multiple provinces, and the geographic distribution showed a declining gradient outward from Shanghai (Fig. 2), reflecting the population "siphon effect" of economically developed regions. This demographic pattern implies that carrier data from metropolitan hubs may reflect broader nationwide trends, rather than local genetic characteristics alone. Therefore, conventional region-based genetic surveillance may be insufficient in the era of high mobility. We propose developing dynamic national genetic maps and multi-center surveillance networks to distinguish primary carrier aggregation from secondary gene flow, thereby facilitating precision public health strategies. The detection of Hb H-risk couples originating from a low-incidence province (Anhui) further supports the role of migration in reshaping genetic disease geography.

Conclusions

In summary, this study represents one of the earliest large-scale carrier screening efforts utilizing LRS. Our findings demonstrate that LRS is a powerful and clinically practical approach for detecting carriers and identifying high-risk couples affected by structurally complex

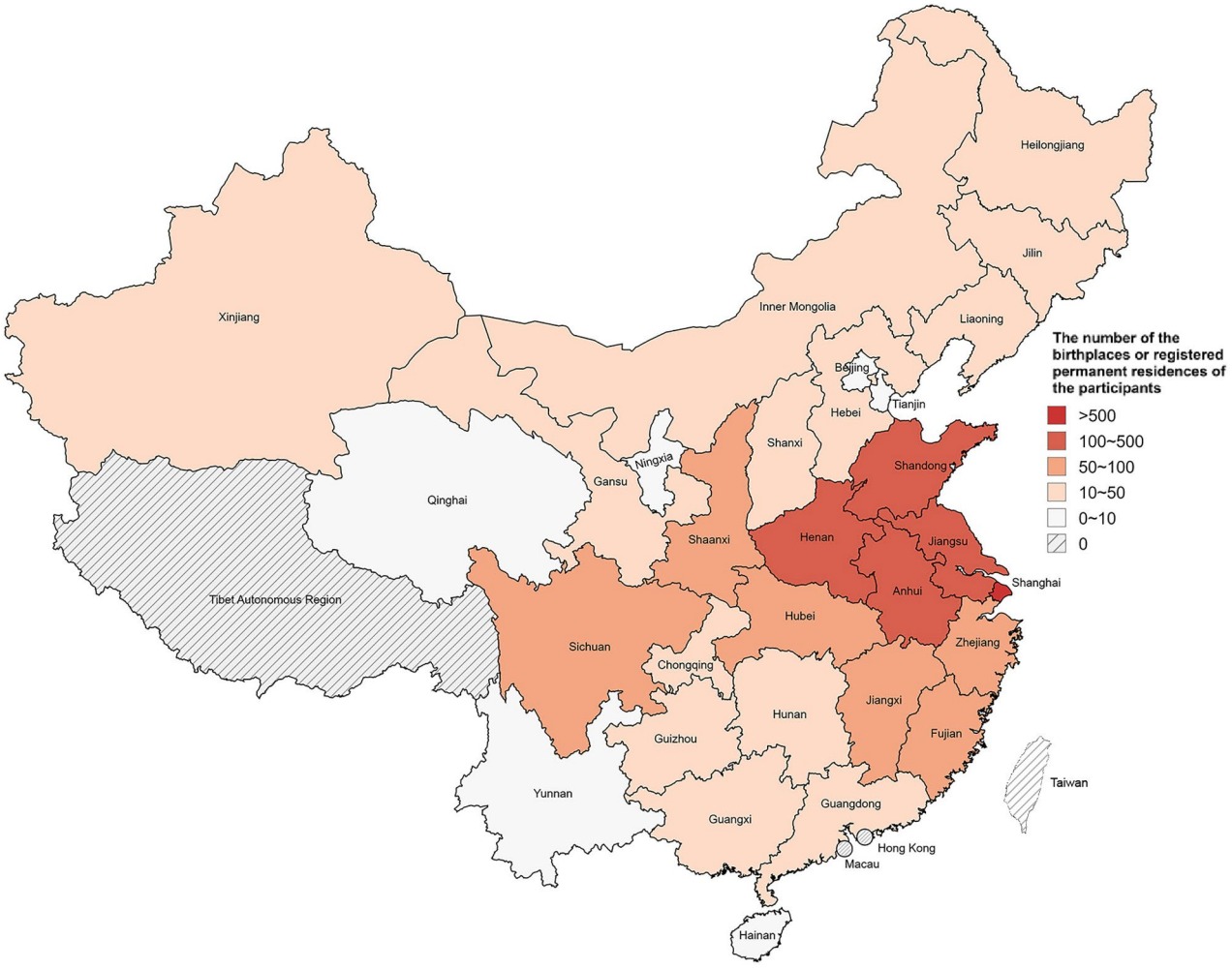

Fig. 2 Geographic origins (birthplaces/registered residences inferred from national ID information) of individuals screened in Shanghai. It is created with MapChart (https://www.mapchart.net/index.html)

monogenic diseases within a premarital population. With superior technical performance, high diagnostic yield, and clear public health value, LRS holds strong potential for implementation in routine premarital genetic screening. We recommend the gradual integration of LRS into routine premarital genetic screening programs to support the primary prevention of birth defects and enhance reproductive health outcomes.

Abbreviations

NGS	Next-generation sequencing
LRS	Long-read sequencing
SMA	Spinal muscular atrophy
CAH	Congenital adrenal hyperplasia
21-OHD	21-Hydroxylase deficiency
FXS	Fragile X syndrome
Hb H	Hemoglobin H
NCCAH	Non-classical CAH
PME	Premarital medical examination
WES	Whole-exome sequencing
WGS	Whole-exome sequencing
qPCR	Quantitative polymerase chain reaction
ARMS-PCR	Amplification refractory mutation system-polymerase chain reaction
SNVs	Single nucleotide variations
indels	Insertions and deletions
CCS	Circular consensus sequencing
ACMG	American College of Medical Genetics and Genomics
HGMD	Human Gene Mutation Database
P	Pathogenic
LP	Likely pathogenic
VUS	Variants of uncertain significance
SW	Salt wasting
SV	Simple virilizing
EMQN	European Molecular Genetics Quality Network
XL	X-linked
PGT-M	Preimplantation genetic testing for monogenic diseases
MLPA	Multiplex ligation-dependent probe amplification
gap-PCR	Gap-polymerase chainreaction
RDB	Reverse dot blot hybridization
SBA	Southern blot analysis
TP-PCR	Triple primer PCR
CE	Capillary electrophoresis
PGT	Preimplantation genetic testing

Supplementary Information

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Supplementary material 1.

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Not applicable.

Author contributions
XH, JW and YW designed the study and drafted the manuscript. RH, SL and DC performed the statistical analysis and wrote the manuscript. YL, YL, LG, SL, and RM performed the sampling and clinical follow up. YL and AM performed sequencing and data analysis. XH, JW and YW reviewed and helped to revise the manuscript. All authors read and approved the final manuscript.

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Data availability
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
This study was approved by the Ethics Committee of International Peace Maternity and Child Health Hospital (#GKLW-A-2024-069-01).

Consent for publication
Written informed consent was obtained from all participants. They all agreed to join the study and publish their clinical and genetic information.

Competing interests
The authors declare no competing interests.

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