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Full length article

Haplotype-Based noninvasive prenatal diagnosis for duchenne muscular dystrophy: A pilot study in South China

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ABSTRACT

Objective: To explore the accuracy and feasibility of noninvasive prenatal diagnosis (NIPD) for Duchenne Muscular Dystrophy (DMD) based on the haplotype approach.

Methods: We recruited singleton pregnancies at-risk of DMD at 12–25 weeks of gestation from 17 families who all had a proband child affected by DMD. We have identified the pathogenic mutations in probands and their mothers by multiplex ligation-dependent probe amplification (MLPA). To construct parental haplotypes, we performed captured sequencing on genomic DNA from parents and probands. The integration analysis of parental haplotypes and targeted sequencing results of maternal plasma DNA were used to infer the fetal haplotype and genotypes in the *DMD* gene. Fetal *DMD* genotypes were further confirmed by invasive prenatal diagnosis.

Result: We have successfully performed the haplotype-based NIPD in all recruited families. Ten fetuses were identified as normal, including four female and six male fetuses. Four female fetuses were carriers, and the other three male fetuses were affected by *DMD* with exons 49–52 deletion, exons 8–37 deletion and c.628 G > T mutation, respectively. The results of NIPD were consistent with those of invasive diagnosis.

Conclusion: Haplotype-based NIPD for DMD by targeted sequencing is promising and has the potential for clinical application.

Introduction

Duchenne Muscular Dystrophy (DMD) is an X-linked recessive genetic disorder affecting 1 in 3500 new-born males. Approximately 70% of DMD patients have at least one exon deletion/repeat, and the others are point mutations or small insertions/deletions of the *DMD* gene. The current standard in prenatal diagnosis is to provide invasive procedures, chorionic villus sampling (CVS) or amniocentesis for genetic study [1]. However, a small risk of miscarriage is associated with invasive procedures [2].

During the last decade, non-invasive prenatal test (NIPT) for aneuploidy using maternal plasma cell-free fetal DNA (cff-DNA) has been widely used in clinical practice. Further research has been conducted to develop NIPD for single gene disorders (SGDs) using various technologies such as real-time PCR, COLD-PCR, digital PCR, cSMART and next-generation sequencing (NGS). The initial attempts were limited to the exclusion of paternal inheritance

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Table 1
Clinical Information and Molecular Diagnosis.

Family	Sample ID	GW	DMD MLPADiagnosis
F01	mother		Het. D.EX46-48
	father		Normal
	proband		Male, D.EX46-48
	plasma	13W + 1D	-
	CV		Female, Het. D.EX46-48
F02	mother		Het. D.EX20-37
	father		Normal
	proband		Male, D.EX20-37
	plasma	18W + 1D	-
	AF		Female, Normal
F03	mother		Het. D.EX7-47
	father		Normal
	proband		Male, D.EX7-47
	plasma	11W + 3D	-
	CV		Male, Normal
F04	mother		Het. D.EX43-45
	father		Normal
	proband		Male, D.EX43-45
	plasma	20W + 1D	-
	AF		Female, Het. D.EX43-45
F05	mother		Het. D.EX45-47
	father		Normal
	proband		Male, D.EX45-47
	plasma	14W + 1D	-
	CV		Female, Normal
F06	mother		Het. D.EX49-52
	father		Normal
	proband		Male, D.EX49-52
	plasma		-
	CV	12W + 1D	Male, D.EX49-52
F07	mother		Het.c.628 G > T
	father		Normal
	proband		Male, c.628 G > T
	plasma	13W + 2D	-
	CV		Male, c.628 G > T
F08	mother		Het. D.EX17
	father		Normal
	proband		Male, D.EX17
	plasma	13W + 2D	-
	CV		Female, Normal
F09	mother		Het. D.EX45-48
	father		Normal
	proband		Male, D.EX45-48
	plasma	19W + 1D	-
	AF		Female, Het. D.EX45-48
F10	mother		Het. D.EX8-37
	father		Normal
	proband		Male, D.EX8-37
	plasma	12W + 1D	-
	CV		Male, D.EX8-37
F11	mother		Het. D.EX54
	father		Normal
	proband		Male, D.EX54
	plasma	12W + 5D	-
	CV		Male, Normal
F12	mother		Het. D.EX48-49
	father		Normal
	proband		Male, D.EX48-49
	plasma	11W + 2D	-
	CV		Female, Normal
F13	mother		Het. Dup.EX49-50
	father		Normal
	proband		Male, Dup.EX49-50
	plasma	12W + 4D	-
	CV		Male, Normal
F14	mother		Het. D.EX46-50
	father		Normal
	proband		Male, D.EX46-50
	plasma	26W + 6D	-
	AF		Male, Normal
F15	mother		Het. D.EX12-20
	father		Normal
	proband		Male, D.EX12-20
	plasma	20W + 3D	-
	AF		Male, Normal
F16	mother		Het.c.1408A > T

Table 1 (*Continued*)

Family	Sample ID	GW	DMD MLPADiagnosis
	father		Normal
	proband		Male, c.1408A > T
	plasma	25W + 4D	-
	AF		Male, Normal
F17	mother		Het. D.EX14-15
	father		Normal
	proband		Male, D.EX14-15
	plasma	11W + 1D	-
	CV		Female, Het. D.EX14-15

Notes: Abbreviations: CV, Chorionic Villli;AF, Amniotic fluid; Het. D.EX, Heterozygous deletion Exon; Y, Years; GW, Gestational Weeks.

[3,4] and detection of de novo mutations [5]. The relative haplotype approach is a perfect solution to detect the maternal inherited alleles and alleles shared by both parents [6–8]. However, establishing an accredited NIPD service is challenging with regards to the cost-effectiveness and quality control of fetal DNA fraction, reads depth of cff-DNA and the informative SNPs [9].

Here we reported the haplotype-based NIPD for DMD in a large sample size.

Material and methods

Patients and sample collection

Seventeen at-risk families with probands (male) were recruited with genetic counseling and informed consent. The study was approved by the institutional Ethics Committee. For each family, we collected 10 ml blood samples from the parents and proband, 5 mg Chorionic Villus (CV) or 10 ml Amniotic Fluid (AF) from the intrauterine cavity. The causative mutation in the *DMD* gene in each family was identified using MLPA (Table 1).

DNA sequencing library preparation

A 657.29Kb SeqCap kit (Roche, Basel, Switzerland) containing 13.91Kb coding region, 3965 Single Nucleotide Polymorphisms (SNPs) (MAF 0.3−0.5) located within the 1 M region flanking *DMD* gene (Fig. 1) and gender determination locus in chromosome Y was designed. Genomic DNA (gDNA) was extracted from blood and AF or CV sample with QIAamp DNA Blood Mini Kit. Cff-DNA was extracted from plasma performing QIAamp Circulating Nucleic Acid kit. Cff-DNA and gDNA library was prepared referred to Kapa Biosystems library preparation kit and Illumina standard protocol, respectively. The post-capture libraries were sequenced by PE 101 bp on Illumina Hiseq2500.

Haplotype-based NIPD for DMD

After sequencing, we mapped the reads to the human reference genome (hg19) by performing the BWA software (version 0.7.12), removed the duplicated reads using Picard Tools and called the SNPs by GATK software after filtering the low-quality reads (mapping quality <13). The variants for further analysis met the following criteria: depth≥50x, quality≥30, allele frequency≥1%. We selected the SNPs that were homozygous in both parents but with different genotypes to calculate the fetal DNA fraction according to the formula: $\varepsilon = 2d_F / (d_F + d_M)$ (ε, fetal DNA fraction; d_F, the depth of father's SNPs; d_M, the depth of mother's SNPs) [10].

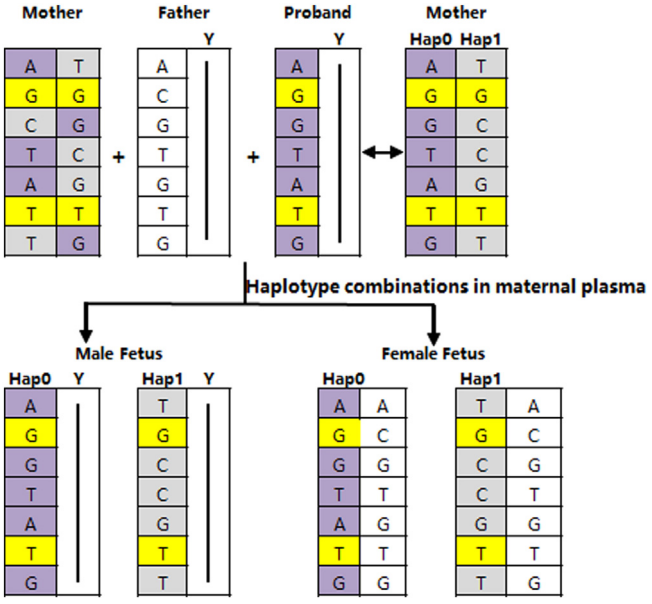

Fig. 2. The haplotype-based approach of NIPD for DMD.

The haplotype linked with mutant and wild allele was constructed using the sequence data of family (Fig. 2). Based on the linkage relationship obtained from parental haplotypes and the base sequence obtained from plasma DNA sequencing, the Hidden Markov Model (HMM) was constructed to deduce the fetal genetic allele of *DMD*. To construct the HMM, we analyzed the SNPs where the mother was heterozygous, and the father was homozygous. For each site, we used the number of reads in maternal plasma to calculate the probabilities that the fetus inherited the pathogenic and non-pathogenic allele. These probabilities were HMM emission probabilities. Recombination rates between SNPs are provided via a genetic map (from NCBI) that specifies the genetic position of the SNPs in cM, and these probabilities were HMM

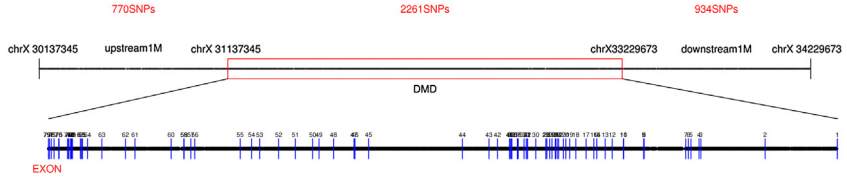

Fig. 1. The target region of the *DMD* gene and SNPs used for haplotyping.

Table 2
Statistics of Target Region Sequencing Data.

Case	Sample ID	Capture specificity	Duplication rate	Depth of target region	Coverage (Depth > = 20X)	Mean depth of target region in ChrY	Coverage ≥4× in ChrY	Type 1 SNPs (n)	Type 2 SNPs (n)	Error rate	cffDNA concentration
F01	mother	42.38%	16.80%	159.79	99.08%	1.50	26.70%				
	father	60.28%	17.93%	149.98	98.82%	105.35	100.00%				
	proband	42.05%	18.08%	159.00	98.75%	112.37	100.00%				
	plasma	41.37%	19.63%	158.92	99.13%	5.87	29.21%	484	470	0.35%	9.30%
	CV	74.16%	45.15%	479.31	99.63%	1.23	25.84%				
F02	mother	47.87%	27.98%	116.24	98.55%	0.00	0.00%				
	father	52.97%	32.29%	125.85	98.66%	81.75	100.00%				
	proband	53.43%	27.69%	106.24	97.73%	68.85	100.00%				
	plasma	53.97%	29.26%	116.45	98.52%	2.49	10.05%	329	592	0.33%	8.50%
	AF	58.08%	33.50%	213.47	99.45%	1.57	22.84%				
F03	mother	69.33%	27.54%	118.81	98.59%	0.00	0.00%				
	father	57.65%	28.44%	97.86	97.81%	64.21	100.00%				
	proband	54.54%	26.92%	94.11	94.37%	63.21	100.00%				
	plasma	49.07%	32.89%	112.57	98.57%	26.33	99.28%	402	221	0.28%	12.77%
	CV	53.45%	32.10%	262.80	99.67%	65.46	100.00%				
F04	mother	51.56%	19.04%	78.25	97.24%	1.13	32.39%				
	father	50.35%	27.03%	137.38	98.86%	91.57	100.00%				
	proband	49.58%	24.59%	86.80	96.45%	56.51	100.00%				
	plasma	53.10%	28.46%	223.09	99.16%	2.63	9.89%	600	410	0.38%	7.83%
	AF	56.59%	39.03%	208.07	99.44%	1.68	41.18%				
F05	mother	53.10%	28.34%	149.61	99.05%	0.00	0.00%				
	father	54.90%	26.45%	83.57	97.17%	56.20	99.86%				
	proband	49.23%	27.00%	78.85	96.33%	52.37	100.00%				
	plasma	52.51%	30.98%	78.09	97.00%	2.06	38.70%	460	312	1.92%	11.15%
	CV	74.09%	19.40%	264.05	99.45%	1.10	16.03%				
F06	mother	57.28%	28.35%	113.72	98.55%	0.00	0.00%				
	father	54.44%	28.35%	98.23	97.98%	66.87	99.98%				
	proband	69.57%	33.59%	106.73	97.72%	73.24	100.00%				
	plasma	55.26%	32.32%	90.35	97.85%	14.11	99.13%	317	269	2.16%	6.22%
	CV	46.40%	22.56%	275.98	99.48%	32.29	100.00%				
F07	mother	57.04%	29.49%	108.09	98.36%	0.00	0.00%				
	father	56.63%	32.15%	128.55	98.81%	84.56	100.00%				
	proband	55.05%	30.52%	106.56	98.17%	68.63	100.00%				
	plasma	53.58%	34.84%	148.73	99.05%	18.45	99.39%	419	450	0.39%	9.42%
	CV	45.98%	26.46%	264.59	99.47%	89.73	100.00%				
F08	mother	54.08%	22.47%	128.75	98.80%	0.00	0.00%				
	father	54.18%	22.45%	117.40	98.52%	77.56	100.00%				
	proband	54.65%	22.75%	125.58	98.61%	80.71	100.00%				
	plasma	48.37%	27.61%	265.35	99.52%	4.75	18.23%	301	590	0.41%	10.25%
	CV	–	–	–	–	–	–			–	
F09	mother	52.74%	25.27%	102.38	98.26%	0.00	0.00%				
	father	50.61%	29.45%	116.09	98.45%	73.64	100.00%				
	proband	45.86%	24.76%	95.38	97.26%	61.80	100.00%				
	plasma	52.43%	28.28%	279.38	99.51%	3.15	10.35%	360	537	0.40%	15.38%
	AF	49.01%	33.06%	112.57	98.57%	4.51	86.79%				
F10	mother	38.78%	36.51%	198.08	99.34%	0.00	0.00%				
	father	37.58%	30.05%	162.07	99.23%	107.06	100.00%				
	proband	49.76%	35.55%	197.03	97.65%	130.84	100.00%				
	plasma	30.19%	18.21%	277.86	99.64%	13.08	99.72%	748	590	0.30%	6.38%
	CV	41.24%	37.91%	256.23	98.03%	155.85	100.00%				
F11	mother	40.96%	37.04%	145.05	99.16%	0.00	0.00%				
	father	38.30%	37.34%	124.11	98.85%	82.62	100.00%				
	proband	41.87%	36.14%	122.62	98.81%	80.96	100.00%				
	plasma	29.73%	18.13%	240.37	99.66%	17.51	97.15%	782	855	0.21%	10.93%
	CV	39.26%	30.58%	238.96	99.72%	129.03	100.00%				
F12	mother	51.02%	34.25%	135.55	99.09%	0.00	0.00%				
	father	51.06%	36.94%	151.68	99.25%	96.51	100.00%				
	proband	49.37%	33.66%	144.49	98.75%	95.01	100.00%				
	plasma	50.80%	30.02%	184.06	99.36%	2.25	8.06%	722	566	0.24%	10.13%
	CV	36.17%	22.14%	227.69	99.59%	2.20	16.28%				
F13	mother	56.53%	36.69%	148.94	99.18%	0.00	0.00%				
	father	53.81%	35.57%	139.62	99.12%	90.04	100.00%				
	proband	46.23%	34.48%	132.11	99.05%	86.90	100.00%				
	plasma	39.51%	14.94%	222.78	99.45%	11.32	97.75%	613	503	1.31%	3.61%
	CV	55.77%	38.95%	232.49	99.65%	144.01	100.00%				
F14	mother	57.70%	30.68%	177.84	99.26%	0.00	0.00%				
	father	52.66%	33.75%	181.26	99.37%	117.31	100.00%				
	proband	51.26%	33.38%	176.19	98.78%	115.78	100.00%				
	plasma	38.53%	16.20%	244.33	99.56%	14.76	100.00%	585	848	0.25%	8.37%
	AF	60.35%	35.68%	277.83	99.72%	184.41	100.00%				
F15	mother	60.04%	31.31%	311.74	99.64%	0.00	0.00%				
	father	59.33%	34.20%	201.97	99.60%	139.65	100.00%				
	proband	59.73%	35.01%	252.17	99.14%	171.05	100.00%				
	plasma	41.59%	26.02%	224.36	99.58%	14.64	98.94%	764	659	0.21%	8.23%
	AF	57.62%	27.36%	186.09	99.44%	130.08	100.00%				

Table 2 (*Continued*)

Case	Sample ID	Capture specificity	Duplication rate	Depth of target region	Coverage (Depth > = 20X)	Mean depth of target region in ChrY	Coverage ≥4× in ChrY	Type 1 SNPs (n)	Type 2 SNPs (n)	Error rate	cffDNA concentration
F16	mother	61.64%	33.26%	228.84	99.52%	0.00	0.00%				
	father	59.79%	26.19%	140.40	99.19%	95.96	100.00%				
	proband	59.66%	27.81%	150.73	99.22%	104.15	100.00%				
	plasma	42.47%	30.95%	210.52	99.69%	23.54	99.82%	603	696	0.25%	16.97%
	AF	58.47%	27.85%	195.25	99.55%	131.34	100.00%				
F17	mother	47.57%	36.85%	195.34	99.43%	0.00	0.00%				
	father	59.96%	32.64%	227.35	99.63%	158.75	100.00%				
	proband	59.96%	31.97%	139.82	99.13%	94.01	100.00%				
	plasma	40.39%	33.09%	201.42	99.55%	2.66	12.20%	627	1001	0.24%	11.19%
	CV	59.18%	28.41%	288.07	99.65%	4.73	55.96%				

Notes: Type 1 SNPs refers to SNPs that were homozygous with the same genotype in both parental genomes were used to calculate the plasma sequencing error rate; Type 2 SNPs refers to SNPs that were homozygous in both parents but had different types and were used to calculate the cell-free fetal DNA (cff-DNA) concentration. The CV sample in F08 family has no sequencing data due to its failed library.

transition probabilities. Finally, we used the Viterbi algorithm to infer the most likely inherited haplotype.

The average depth and coverage of the specific region on the Y chromosome was used to infer fetal gender. Plasma samples of male fetus demonstrated four times higher coverage on the target region of the Y chromosome and higher mean depth but almost no reads mapped to Y specific region in the female fetus. Thus, the sex of the fetus could be determined based on the average depth and coverage of the particular region of the Y chromosome.

Validation of NIPD for DMD

The *DMD* genotype of the fetal sample obtained by invasive procedure was confirmed by MLPA (SALSA P021 and P060 DMD probe mix, Netherlands) blindly. The AF or CV sample was also captured sequencing using the same probe to prove the accuracy of NIPD further. To evaluate the accuracy of NIPD under different sequencing depth/fraction/informative of SNPs, a series of computer simulation experiment was executed by comparing the inferred SNPs with the fetal genotyping.

Results

The clinical information of seventeen families including the fetal genotype was shown in Table 1. *DMD* mutations in each family including large fragment deletions and point variants are summarized in Table 1.

After sequencing and bioinformatics analysis, a mean of 152X and 248X reads were obtained for gDNA and cell-free DNA, respectively (Table 2). The mean (± SD) capture specificity, 20X coverage, cffDNA concentration and duplication rate in the target region were 51.60% (±8.62%), 98.83% (±0.92%), 9.80% (±3.16%) and 29.39% (±6.09%), respectively (Table 2).

In F03, F06, F07, F10, F11, F13, F14, F15 and F16, the mean depth of specific- region on the Y chromosome was about 18X (range: 11.32–26.33). The region with reads coverage at least four was over 97% (range: 97.15–100%), indicating male fetuses. In the remaining female fetuses, the mean depth of specific region on the Y chromosome ranged from 2.06 to 5.87 with 4X coverage being 8.06%–38.70%. These results were confirmed by the sequencing data of fetal genomic DNA.

We constructed fetal haplotypes for each fetus by using 348–977 informative SNPs phased on the target region. NIPD results revealed four normal female fetuses (F02, F05, F08 and F12), six normal male fetuses (F03, F11, F13, F14, F15 and F16), four female carriers (F01, F04, F09 and F17), and three affected male fetuses (F06, F07 and F10) (Table 3 and Fig. 3). The result was in concordant with invasive diagnosis using MLPA.

To further evaluate the inferred fetal haplotype, we performed target capture sequencing in CV or AF samples (failed in the CV in F08). Fetal haplotypes constructed using the parental-fetus genomic DNA sequencing data were the same as the corresponding fetal haplotypes inferred through plasma DNA sequencing. There were no false positive/negative results.

Table 3
Noninvasive Prenatal Test of DMD Families.

Family	Fetal Gender	Phased SNPs[a]	SNPs For M0[b]	SNPs For M1[c]	Inherited Maternal	NIPT-based Predicted testing results	MLPA diagnosis
F01	Female	563	560	0	M0	Carrier	Het. D.EX46-48
F02	Female	554	0	551	M1	Normal	Normal
F03	Male	445	0	444	M1	Normal	Normal
F04	Female	402	234	168	M0	Carrier	Het. D.EX43-45
F05	Female	469	29	440	M1	Normal	Normal
F06	Male	442	441	0	M0	Affected	D.EX49-52
F07	Male	631	629	0	M0	Affected	Hem. c.628 G > T
F08	Female	585	0	582	M1	Normal	Normal
F09	Female	434	310	123	M0	Carrier	Het. D.EX45-48
F10	Male	610	350	253	M0	Affected	D.EX8-37
F11	Male	905	231	654	M1	Normal	Normal
F12	Female	424	0	423	M1	Normal	Normal
F13	Male	783	275	505	M1	Normal	Normal
F14	Male	977	0	962	M1	Normal	Normal
F15	Male	599	13	575	M1	Normal	Normal
F16	Male	529	0	527	M1	Normal	Normal
F17	Female	348	339	0	M0	Carrier	Het. D.EX14-15

Notes: M0, fetal-inherited maternal mutant haplotype; M1, fetal-inherited maternal wild-type haplotype.
SNPs[a] represents SNPs that were used to predict maternal haplotypes with a trio strategy.
SNPs[b] represents number of SNPs supported for M0.
SNPs[c] represents number of SNPs supported for M1.

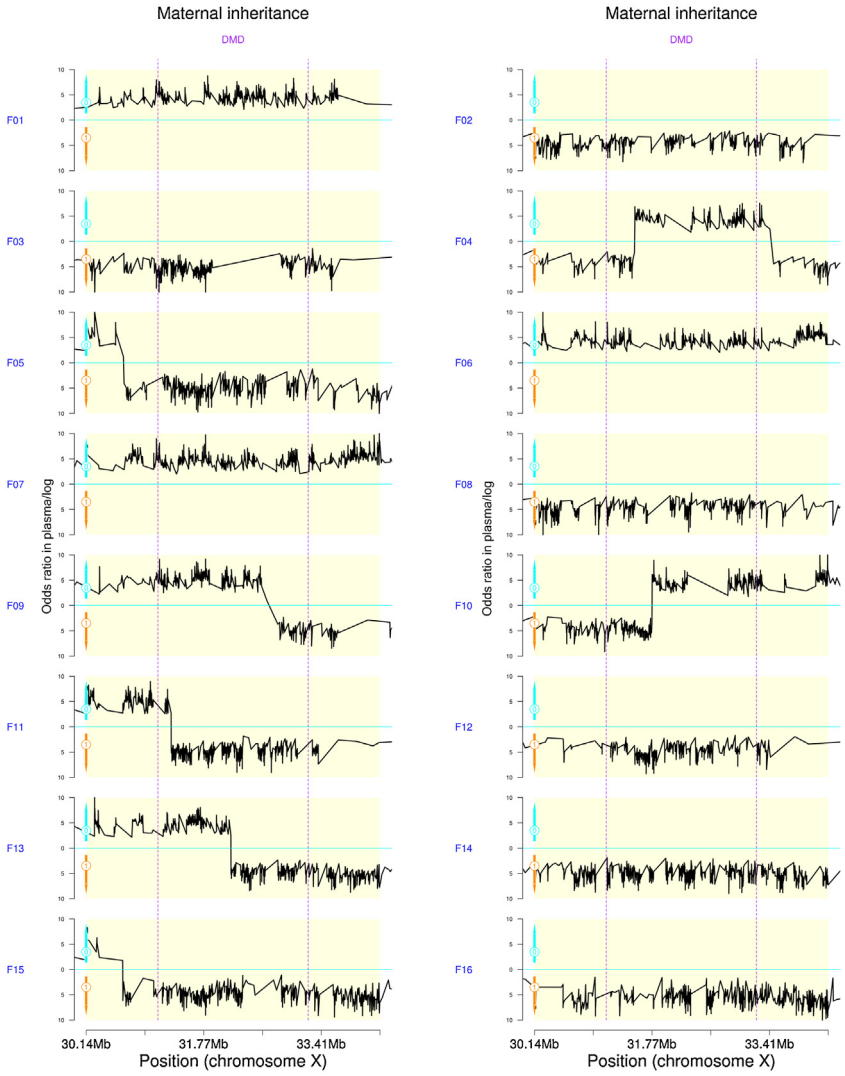

Fig. 3. Fetal haplotype inference. The X-axis represents the locus on chromosome X; The Y-axis represents the logarithm of the ratios of fetal different haplotype combinations; The blue Line fetus inherited from maternal haplotypes. Lines above zero (Cyan lines) indicate that the fetus inherited the pathogenic allele (Hap0), and the lines below zero point to that the fetus inherited the normal allele (Hap1) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Discussion

In this study, all 17 fetuses were accurately diagnosed as the normal, carrier or affected using haplotype-based NIPD. This study and previous works of NIPD for DMD showed a high degree of accuracy compared with the invasive diagnosis results, as shown in Table 4 [11–14]. We also reviewed NIPD for other monogenic disorders [15]. The accuracy of NIPD for SGDs was near 100% [6,7,14,16–21] (Table 5).

Although technically possible, the high cost of NIPD may be a significant factor limiting its clinical application. The price is about 600–900 dollars for one sample based on an independent experimental operation. The cost is mainly composed of high depth sequence, the commercial capture probe and bespoke design service for specific gene and mutation uncovered an existing probe. The small sample size in one turn round of NIPD would also increase the cost. In our work, several attempts have been made to reduce cost. First, the capture region of the customized probe was narrowed to 657.29 kb including the coding region and flanking region in the *DMD* gene. Second, 100X sequence depth was determined to be cut off to balance the relationships between sequence cost and accuracy. Third, explore

Table 4
Studies reporting the accuracy of non-invasive prenatal testing of DMD.

Author	Country	Design	Objects	Diagnosis method	Case No.	Accuracy
Yan Xu	China	Prospective Study	DMD families	Sanger /QPCR	8	100%
Michel Parks	UK	Prospective Study	DMD/BMD families	MLPA	9	100%
Seong-Keun Yoo	Korea	Prospective Study	DMD families	MLPA/Sanger	4	100%
Current Study	China	Prospective Study	DMD families	MLPA/Sanger	17	100%
Total					38	100%

Notes: Only first author is given for each study. No. means number.

Table 5
The accuracy of NIPT for other single-gene diseases.

Diseases	Gene	Case	NIPT	Concordant rate
Alpha-thalassemia	*HBA1*, *HBA2*	1	1	100%
Beta-thalassemia	*HBB*	2	2	100%
Phenylketonuria (PKU)	*PAH*	13	12	100%
Methylmalonic acidemia	*MMACHC*	19	6	100%
Congenital adrenal hyperplasia	*CYP21A2*	12	12	100%
Spinal Muscular Atrophy (SMA)	*SMN1*	14	13	100%
Duchenne/Becker muscular dystrophy (DMD/BMD)	*DMD*	17	17	100%
Hemophilia A	*F8*	1	0	100%
Hemophilia B	*F9*	1	1	100%
Hereditary hearing impairment	*GIB2*	1	1	100%
Total		83	67	

the maximization of samples on the same probe capture reaction. In our study, the best experimental scheme was to recruit samples of five families, each of which included maternal cff-DNA, the parental and proband's genomic DNA (gDNA). The capture probe could be expanded to more monogenic diseases with higher incidence to reduce the bespoke design cost. With the development of technology [22], the cost of sequencing will continue to decrease.

The sequencing depth, fetal fraction and number of informative SNPs are vital factors to ensure the accuracy and reliability of NIPD in clinical service. Our study innovatively elaborated quality control using computer simulation. When the fetal fraction is below 5%, the number of corresponding SNPs required for fetal haplotype constructing is at least 40 to reach the accuracy of 99%. When the plasma sequence depth is 200X, the accuracy of the inferred fetal haplotype is close to 100% (Supplemental Fig. S1). Fetal fractions of other families were all above 5%, the informative SNPs were all above 200, and the accuracy of NIPD was 100%. If the informative SNPs were enough to predict the fetal haplotype, we could reduce the plasma sequence depth to cut the cost. So, we evaluated the effect of sequencing depth on inferring fetal haplotypes (Supplemental Fig. S2). If the sequencing depth of plasma was reduced to 100X, 20 or more SNPs were used to infer fetal-maternal haplotypes to achieve an accuracy of 99%. If the sequencing depth of plasma was reduced to 60X, at least 35 SNPs was needed to predict the fetal inherited maternal haplotype to ensure accuracy of 99%. The accuracy depended on the number of informative SNPs affected by sequence depth of plasma and fetal DNA fraction. Therefore, the cut-off must be determined by considering the interaction of three factors. According to the computer simulation experiments, to ensure the detection accuracy with 99%, the cutoff value of the number of informative SNPs was 20, the deduced depth of plasma was prescribed as 100X and the fetal DNA fraction was defined to be 5%.

Due to the recombination frequency of the entire dystrophin gene can be as high as 12% [23], it is crucial to infer the fetal inherited allele for recombination accurately. Currently, the position of the recombination event was confirmed by comparing the haplotypes of the proband and the CVS, or by bi-directional (*i.e.*, 5′to 3′and 3′to 5′) evaluation of recombination sites in RHDO analysis [16]. We precisely assessed the position of the combination site to support a correct diagnosis, demonstrating the robustness of the haplotype-based approach. Nevertheless, further improvements are necessary. Establishing referral laboratories for DMD with increased multiplexing capacity of multi-personnel in each sequencing run will help to reduce the cost and shorten the turn-around-time [16].

Recently, linked-reads sequencing technology based on microfluidics has become available, allowing direct haplotype phasing of the target region and NIPD were successfully achieved [13]. This approach does not rely on the availability of DNA from the affected proband and should be accessible to more couples. However, the cost of linked-reads technology was three times as our method due to its expensive library kits and equipment. The 10x Genomics technology was more labor intensive and not as scalable as our method. Its application in the high-risk pregnancy for DMD needs further study.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ejogrb.2019.05.005.

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