Clinical Chemistry 61:6
829–837 (2015)

Noninvasive Prenatal Diagnosis of Duchenne Muscular Dystrophy: Comprehensive Genetic Diagnosis in Carrier, Proband, and Fetus

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BACKGROUND: Noninvasive prenatal diagnosis of monogenic disorders using maternal plasma and targeted massively parallel sequencing is being investigated actively. We previously demonstrated that comprehensive genetic diagnosis of a Duchenne muscular dystrophy (DMD) patient is feasible using a single targeted sequencing platform. Here we demonstrate the applicability of this approach to carrier detection and noninvasive prenatal diagnosis.

METHODS: Custom solution-based target enrichment was designed to cover the entire dystrophin (*DMD*) gene region. Targeted massively parallel sequencing was performed using genomic DNA from 4 mother and proband pairs to test whether carrier status could be detected reliably. Maternal plasma DNA at varying gestational weeks was collected from the same families and sequenced using the same targeted platform to predict the inheritance of the *DMD* mutation by their fetus. Overrepresentation of an inherited allele was determined by comparing the allele fraction of 2 phased haplotypes after examining and correcting for the recombination event.

RESULTS: The carrier status of deletion/duplication and point mutations was detected reliably through using a single targeted massively parallel sequencing platform. Whether the fetus had inherited the *DMD* mutation was predicted correctly in all 4 families as early as 6 weeks and 5 days of gestation. In one of these, detection of the recombination event and reconstruction of the phased haplotype produced a correct diagnosis.

CONCLUSIONS: Noninvasive prenatal diagnosis of DMD is feasible using a single targeted massively parallel sequencing platform with tiling design.

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Discovery of the presence of cell-free fetal DNA (cffDNA)[10] in maternal plasma offers a powerful tool for the development of noninvasive prenatal genetic diagnosis *(1)*. The application to prenatal diagnosis has been accelerated by the introduction of massively parallel sequencing technology *(2, 3)*. Prenatal tests capable of detecting aneuploidies using cffDNA have been commercialized and are highly sensitive and accurate *(4)*. Several studies have confirmed the accuracy of whole-genome sequencing and sequencing after target enrichment of cffDNA by demonstrating the relatively even distribution of fetal and maternal DNA across the entire genome *(5–7)*. This result provides the basis for extending the applications to monogenic disorders, which comprise a larger proportion of genetic diseases than chromosomal aneuploidies.

However, unlike the rapid incorporation of aneuploidy detection into clinical practice, the application to monogenic disorders is far more complex and has many obstacles to overcome. Technically, the low and variable

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Received November 18, 2014; accepted March 9, 2015.
Previously published online at DOI: 10.1373/clinchem.2014.236380

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[10] Nonstandard abbreviations: cffDNA, cell-free fetal DNA; DMD, Duchenne muscular dystrophy; IRB, institutional review board; indel, small insertion and deletion; GATK, Gene Analysis Toolkit; SNV, single-nucleotide variant; HapA, haplotype A; bcp, Bayesian change point; DMD-02-9-wk, DMD-02 at 9 weeks; cM, centimorgan.

Table 1. *DMD* mutation status of the study cohort and types of samples sequenced.

Study number	*DMD* mutation (by MLPA[a] or Sanger sequencing)		Genomic DNA sequencing		Maternal plasma DNA sequencing (gestational age)	Fetal DNA sequencing
	Proband[b]	Mother	Proband	Mother		
DMD-01	Exon 49–52 deletion	Carrier	+	+	+ (6 weeks 5 days, 17 weeks 1 day)	+
DMD-02	Exon 2 duplication	Carrier	+	+	+ (9 weeks 3 days, 12 weeks 1 day)	+
DMD-03	Exon 3–7 deletion	Carrier	+	+	+ (8 weeks 5 days, 11 weeks 3 days)	+
DMD-04	c.649 + 2T>C	Carrier	+	+	+ (7 weeks 1 day)	+

[a] MLPA, multiple ligation-dependent probe amplification.
[b] All probands were male.

fraction of cffDNA in maternal plasma limits the reliable detection of fetal variants at the single-nucleotide level. Moreover, complex ethical and socioeconomic issues limit the implementation of noninvasive genome-wide screening in the prenatal diagnosis of monogenic disorders in pregnant women without a known increased risk. Therefore, for clinical applications, the ideal platform needs to be a targeted design that can ensure deep coverage and be equally applicable to the proband and carrier, and for prenatal diagnosis.

We have developed a method that allows the comprehensive genetic diagnosis of a Duchenne muscular dystrophy (DMD) patient. We have shown that this method is feasible when used with a targeted massively parallel sequencing platform *(8)*. Targeting the entire exonic and intronic regions produced nearly continuous uniform coverage across the dystrophin (*DMD*)[11] gene, enabling identification of both large deletions/duplications and point mutations. Because this method is sensitive enough to detect a dosage imbalance, the mother's carrier status could be easily identified with the same approach. In addition, because approximately 1000 heterozygote sites can be used to analyze maternal X alleles at 2 phases, this method may be also applicable to prenatal diagnosis using cffDNA by detecting haplotype imbalances between 2 phased haplotypes in the *DMD* gene. This haplotype-based imbalance analysis via either whole-genome sequencing or targeted sequencing of maternal plasma DNA has been substantiated in models of several diseases, including β-thalassemia, congenital adrenal hyperplasia, and congenital deafness *(5, 9–11)*. Specifically, New et al. adopted a similar approach using targeted sequencing and a tiling design for the noninvasive prenatal diagnosis of congenital adrenal hyperplasia inherited with an autosomal recessive pattern *(11)*.

[11] Human genes: *DMD*, dystrophin; *ZFX*, zinc finger protein, X-linked; *ZFY*, zinc finger protein, Y-linked; *F8*, coagulation factor VIII, procoagulant component.

In the present study, we attempted to demonstrate the feasibility of using the targeted massively parallel sequencing platform for carrier detection and noninvasive prenatal diagnosis of DMD.

Materials and Methods

PATIENTS

The 4 DMD families receiving a prenatal diagnosis were prospectively recruited. Each family cohort consisted of a proband and the carrier mother. *DMD* mutations in the families included both large deletion/duplication and point mutations (Table 1). The experiment was designed and performed in 2 parts. First, genomic DNA from 4 mother and DMD proband pairs was sequenced to test whether an inherited *DMD* mutation from the carrier mother could be detected confidently. Second, maternal plasma DNA from the 4 carrier mothers at varying weeks of gestation was sequenced to determine whether inheritance of a *DMD* mutation from a carrier mother could be predicted in her fetus. Fetal genomic DNA obtained from either chorionic villi sampling or amniocentesis was used to validate the results of the maternal plasma DNA sequencing. All procedures were performed as routine prenatal diagnosis. Additional informed consent was obtained for the study that used maternal plasma DNA and fetal DNA. The institutional review board (IRB) approved the study protocol (IRB no. 1302-055-464).

TARGET ENRICHMENT AND MASSIVELY PARALLEL SEQUENCING

Maternal plasma (8–10 mL) was obtained as described previously *(12)*. To construct the DNA library, we used the SureSelectXT reagent kit (Agilent Technologies) and 0.5–1 μg of plasma DNA for each case. Because the library-preparation section in the SureSelect protocol was designed primarily for genomic DNA, we modified it by diluting all reagents in the kit to prepare the plasma DNA library. This protocol was better suited for small amounts

of input DNA. The adapter-ligated DNA was purified directly with the spin columns provided in the QIAquick PCR purification kit (Qiagen) without further size selection. Four-cycle PCR and SureSelect primers were then used to amplify the adapter-ligated DNA.

We quantified the DNA libraries using a Qubit 2.0 fluorometer (Invitrogen), and we used the DNA 1000 kit with a 2100 bioanalyzer (Agilent) to check the size distribution of the libraries. We generated 0.3–0.5 μg of an amplified plasma DNA library for each sample, with an approximate mean size of 270 base pairs. Targeted sequence enrichment was performed using the SureSelect custom kit (Agilent). The custom capture probes targeted entire transcribed *DMD*, zinc finger protein, X-linked (*ZFX*), and zinc finger protein, Y-linked (*ZFY*) regions according to 4 gene databases (RefSeq, Ensembl, CCDS, and GENCODE) and were designed using Agilent SureDesign (https://earray.chem.agilent.com/suredesign). The following parameters were used for the capture design sequences: density, 2; masking, least stringent; and boosting, balanced. We incubated 300 ng of the amplified plasma DNA library with the capture probes for 24 h at 65 °C, in accordance with the manufacturer's instructions. After hybridization, we selected the captured targets by pulling down the biotinylated probe–target hybrids with streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin; Invitrogen) and purified the targets using a MinElute PCR purification kit (Qiagen). Finally, we enriched the targeted DNA libraries using 12-cycle PCR amplification with SureSelect PCR primers (Agilent). The PCR products were purified using the QIAquick PCR Purification Kit. The library was paired-end sequenced on the Illumina HiSeq 2000 sequencing system. The sequenced paired-end reads were submitted to the EBI European Nucleotide Archive (ENA) database with accession number PRJEB7629 (direct access: http://www.ebi.ac.uk/ena/data/view/PRJEB7629).

VARIANT CALLING

Paired-end sequencing reads were aligned to the human genome (Genome Reference Consortium Human Reference 37) with Bowtie2 aligner (v.2.2.3) *(13)*. Picard Tools (http://picard.sourceforge.net) was used to remove PCR-duplicated reads, and duplicate-free BAM files were indexed by using SAMtools (v.0.1.19) *(14)*. Local realignment around small insertions and deletions (indels) and base quality score recalibration were achieved using the Genome Analysis Toolkit (GATK, v.3.2–2). Variant calling was performed using GATK HaplotypeCaller. We filtered out low-quality variant calls using GATK VariantFiltration with parameters described by GATK Best Practice (http://www.broadinstitute.org/gatk/guide/best-practices) *(15)*. Using our in-house script, we also filtered out variants with a genotype quality ≤30 and read depth ≤200. Lastly, we used ANNOVAR to annotate the unfiltered variants against the RefSeq gene set *(16)*.

STRUCTURAL VARIATION DETECTION

Pindel (0.2.4.w) was used to detect structural variations *(17)*. Only structural variations with a supportive read count ≥50 and minimum length ≥1000 on the *DMD* gene were selected as pathogenic candidates. Compared with coverage plots visualized by the UCSC genome browser, large deletions/duplications were confirmed *(18)*.

HAPLOTYPE CONSTRUCTION

Because of hemizygosity in males, we directly phased the maternal haplotypes of the *DMD* region. Using heterozygous single-nucleotide variants (SNVs) in the genomic DNA sequencing from the carrier mothers and probands, we classified the inherited haplotype that contained a deleterious mutation as haplotype A (HapA) and the other haplotype without a mutation as haplotype B (HapB).

MEASUREMENT OF FRACTIONAL FETAL DNA CONCENTRATION

In addition to the *DMD* gene, capturing the *ZFX* and *ZFY* genes provided a measurement of the fractional fetal DNA concentration. Using mean read depth of 2 zinc finger genes ($\overline{ZFX}$ and $\overline{ZFY}$) with a minimum mapping quality score of 20 and base quality score of 20, we calculated the fractional fetal DNA concentration as:

$$\text{Fractional fetal DNA concentration} = \frac{2 \times \overline{ZFY}}{\overline{ZFX} + \overline{ZFY}} \times 100\%.$$

FETAL GENOTYPE PREDICTION

The *DMD* gene is known to have a high recombination rate, and tests to detect the recombination event and recombination point were performed before fetal genotype prediction. To prevent the occurrence of a prediction error for the recombination point because of outlier values originating from duplicated or repetitive regions, we used the R package 'qcc' for outlier removal *(19)*. After outlier detection, we predicted the change point in the read fraction values using the R package 'bcp' (Bayesian change point) *(20)*. After detection of the recombination event, we reconstructed haplotypes with and without deleterious *DMD* mutations and designated these as HapA* and HapB*, respectively. Subsequently, we predicted the fetal genotype by identifying the allele fraction imbalance between 2 haplotypes obtained from maternal plasma sequencing. Because the inherited allele would be overrepresented in relation to the fetal DNA fraction in the maternal plasma, the fetal genotype was

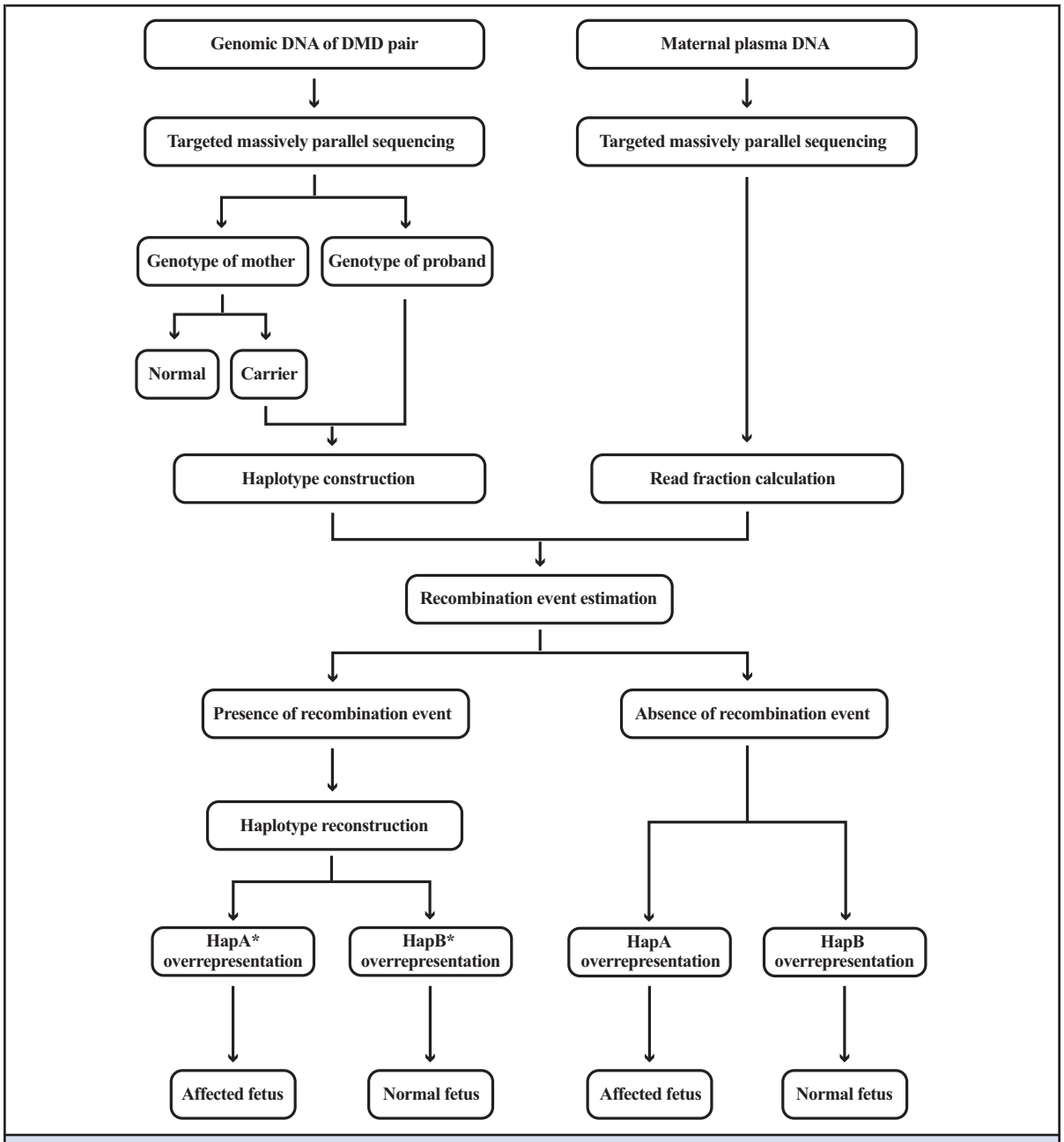

Fig. 1. Work flow of a comprehensive genetic diagnosis in a DMD carrier, proband, and fetus.

determined by estimating which haplotype was overrepresented. If the overrepresented haplotype was the one harboring the *DMD* mutation, the fetus could be predicted to have inherited the *DMD* mutation (Fig. 1).

The statistical significance of the allele fraction imbalance was estimated using a 1-tailed Student's paired *t*-test or Wilcoxon signed-rank test depending on the assumption of normality. All statistical tests were performed with outlier-removed datasets.

Results

CARRIER DETECTION FROM GENOMIC DNA SEQUENCING OF MOTHER AND PROBAND PAIRS

Targeted deep sequencing of 4 mother and proband pairs revealed uniform coverage across the *DMD* gene. A summary of the basic sequencing of the 4 pairs is provided (see Table 1 in the Data Supplement that accompanies the online version of this report at http://www.

clinchem.org/content/vol61/issue6). Large deletions/duplications were identified on visual inspection from a coverage plot across the *DMD* gene in both probands and carrier mothers (see online Supplemental Fig. 1). The breakpoints were estimated successfully using the structural variation detection software Pindel (see online Supplemental Table 2). With the use of visual inspection and breakpoint estimation, the predicted deleted or duplicated exons in all pairs were identical to the previous results detected using the multiple ligation-dependent probe amplification method. In agreement with the previous result, probands with a deletion mutation had nearly zero read depth at the deletion site, whereas the carriers with a deletion mutation had about half the read depth compared with the baseline read depth outside the deleted region (DMD-01 and DMD-03). The read depth height of the carrier with a duplication mutation was positioned between that of the baseline and proband with a duplication mutation (DMD-02). An inherited splice site mutation was also identified in the DMD-04-proband and the carrier mother (see online Supplemental Fig. 2). The number of heterozygous or hemizygous SNVs in carriers and probands ranged from 700 to 1200 (see online Supplemental Table 3). We successfully constructed 2 maternal haplotypes in the *DMD* gene using heterozygous SNVs and their proband haplotype. The mean read depth ratio of *ZFY* to *ZFX* ranged from 0.95 to 0.98 in male probands and was 0 in female carriers, indicating that these zinc finger genes could be used as a reliable indicator of the fractional cffDNA concentration in the subsequent study using maternal plasma (see online Supplemental Table 4).

FETAL GENOTYPE PREDICTION BY MATERNAL PLASMA DNA SEQUENCING

Seven plasma DNA samples obtained from 4 pregnant carriers at different gestational weeks were sequenced. The sequencing results showed uniform and high coverage across the *DMD* gene (see online Supplemental Fig. 3 and online Supplemental Table 1). The number of SNVs and indels were also compatible with the genomic DNA sequencing data (see online Supplemental Table 3). Fractional cffDNA concentration estimated by calculating the mean read depth of *ZFX* and *ZFY* ranged from 5.8% to 9.7% (see online Supplemental Table 4). Higher duplicated read rates (18%–28%) were noted in 7 plasma samples used for DNA sequencing (see online Supplemental Table 5). This difference may have originated from additional PCR cycles used during library preparation and target enrichment because of the low concentration of the input plasma DNA.

Before examining the haplotype imbalance between the 2 phased maternal haplotypes, we investigated the recombination event within the *DMD* region using the R package, as described in Materials and Methods. The bcp algorithm estimated a significant change point in the read fraction in the sequencing data from DMD-02 at 9 weeks (DMD-02-9-wk) and DMD-02-12-wk, which was also evident from a scatterplot of the read fraction distribution of the phased haplotype (Fig. 2). Subsequent analysis suggested the recombination point between chromosomal X positions 32 321 115 and 32 346 373 based on the bcp algorithm. The haplotypes of DMD-02-9-wk and DMD-02-12-wk sequencing data were reconstructed using the recombination point information. Because all the other plasma sequencing data revealed 1 large segment of 2 haplotypes, all remaining families, with the exception of DMD-02, used the same haplotype that was phased from the proband sequencing data (see online Supplemental Fig. 4, A–C). We next attempted to predict the fetal genotype by comparing the allele fraction between the 2 haplotypes in the maternal plasma. In both DMD-01-6-wk and DMD-01-17-wk, the allele fraction of HapB was significantly higher, indicating inheritance of the nonmutated haplotype by the fetus (Fig. 3A). All remaining samples supported the inheritance of a mutated haplotype by the fetuses, including DMD-02-9-wk and DMD-02-12-wk, the haplotypes of which were reconstructed based on a recombination event prediction (Fig. 3, B–D). All the allele fraction differences and statistical test results are provided (see online Supplemental Table 6). The fetal genotypes predicted from the 4 DMD families matched exactly the fetal genomic DNA sequencing data (see online Supplemental Fig. 5 and online Supplemental Table 2).

Discussion

In the present study, we demonstrated that targeted deep sequencing makes feasible not only genetic diagnosis of a DMD patient but also carrier detection and noninvasive prenatal diagnosis. Considering that the prenatal diagnosis of monogenic disorders is still performed in a family-based setting in which a genetically confirmed proband or carrier has been identified, this method has a practical advantage that proband diagnosis, carrier detection, and noninvasive prenatal diagnosis can be accomplished efficiently with a single platform.

For clinical implementation of noninvasive prenatal diagnosis of X-linked recessive disorders including DMD, refining the detection method of dosage imbalance caused by presence of a small fraction of cffDNA is a critical step in identifying the maternally derived genotype. Tsui et al. used digital PCR to detect the slight overrepresentation of a coagulation factor VIII, procoagulant component (*F8*) mutation in pregnant women who are carriers of hemophilia mutations *(12)*. The relative mutation dosage analysis based on a sequential probability ratio test was used. Although it is simple and does not require haplotype information, the detecting

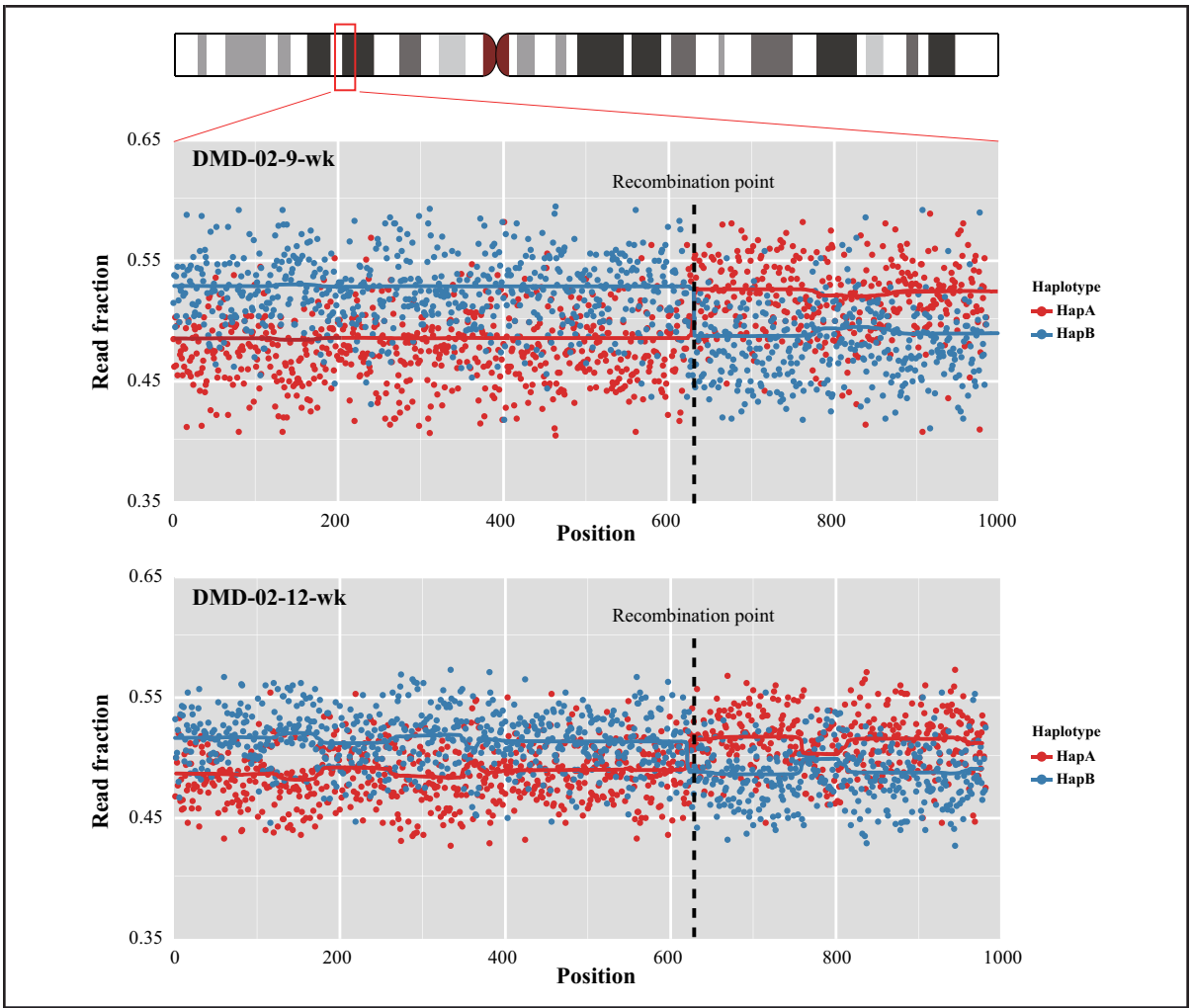

Fig. 2. Detection of a recombination event and haplotype reconstruction in the DMD-02 family.

Read fraction distribution of 2 haplotypes. Each haplotype was divided into 2 large segments. The black dotted lines indicate the recombination point predicted by the bcp algorithm. The karyogram representing chromosome X was generated by ggbio [Yin et al. *(24)*].

probe must be individualized according to the specific mutation type. Further, multiple tests of each sample should be conducted to obtain sufficient statistical power, especially when the fetal DNA fraction is low, as in the early gestational weeks. Relative haplotype dosage analysis may be an alternative option for identifying the slight overrepresentation of an inherited maternal mutation or allele because the genome-wide or targeted massively parallel sequencing approach can produce many informative SNVs for haplotyping. Lam et al. used this approach to identify the maternal inheritance of a mutation in a β-thalassemia model *(9)*. Although β-thalassemia is a disease with autosomal recessive inheritance, a method that identifies the maternal inheritance pattern would be equally applicable to X-linked recessive diseases. Instead of using the separate haplotyping method employed by Lam et al., New et al. sequenced a parent and patient trio, and then used the resulting haplotype information for maternal DNA analysis in the targeted platform used in the trio analysis *(11)*. This diagnostic flow could be incorporated more easily into the current genetic diagnosis and counseling process.

Several gene-specific factors inherent to *DMD* should be considered for clinical implementation of noninvasive prenatal diagnosis. First, large deletion/duplication mutations constitute about two-thirds of the *DMD* mutation spectrum. Because a dosage imbalance already exists in DMD carriers with a large deletion/duplication mutation, it would be difficult to perform relative mutation dosage analysis using digital PCR. Thus, measuring the haplotype imbalance of the *DMD* region outside the mutation would be plausible. Second, the recombination

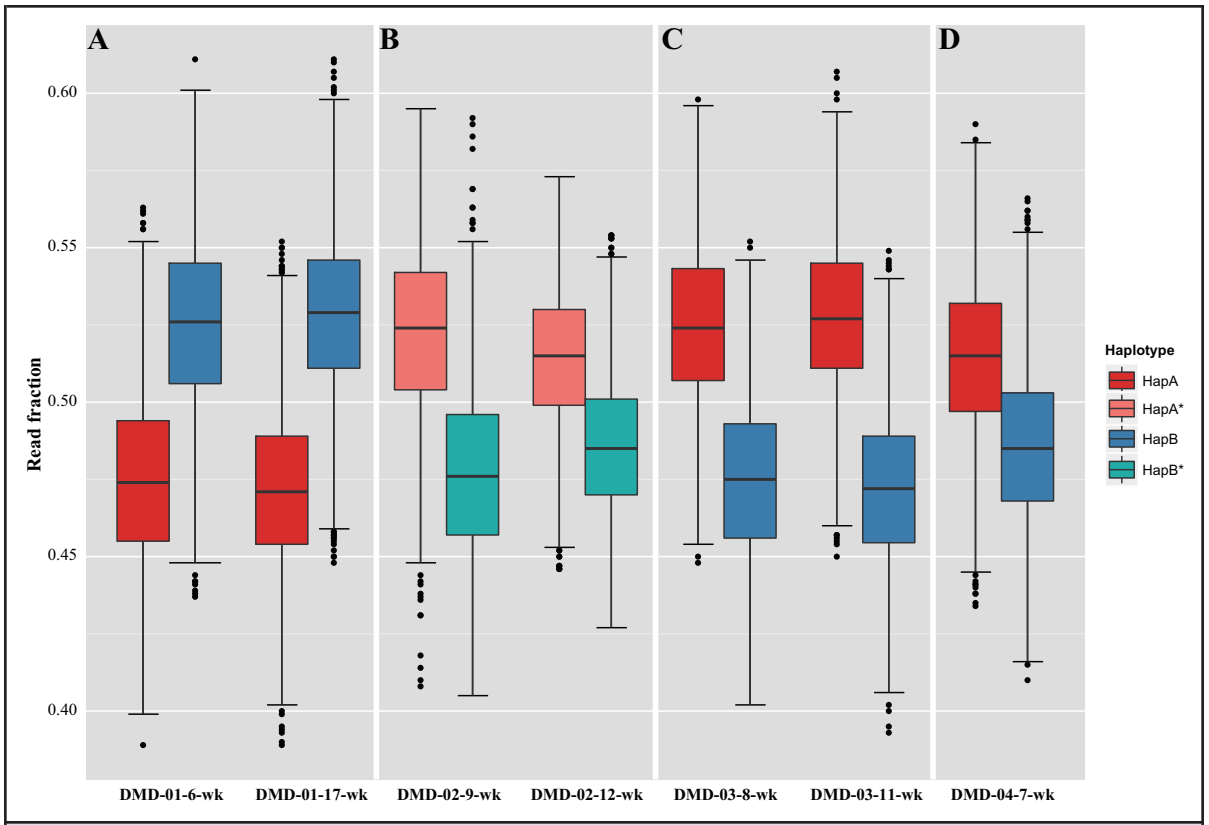

Fig. 3. Fetal genotype prediction.

(A), HapB is overrepresented in DMD-01 maternal plasma samples (allele fraction differences: 5.0% and 5.6%). HapA or HapA* are overrepresented in the remaining maternal plasma samples. (B), DMD-02 (allele fraction differences: 4.4% and 2.9%). (C), DMD-03 (allele fraction differences: 5.6% and 4.9%). (D), DMD-04 (allele fraction difference: 2.9%).

rate of *DMD* is about 4 times higher than the recombination rate with chromosome X and with the whole genome: 4.73 centimorgans (cM)/Mb, 1.21 cM/Mb, and 1.26 cM/Mb, respectively *(21, 22)*. If a recombination event occurred within the *DMD* region, it would greatly affect the dosage imbalance analysis and could result in an incorrect prediction. Fetal genotype prediction without considering the inheritance of recombinant haplotypes would cause nonsignificant haplotype imbalance or contradictory result; it is possible to misdiagnose a non-DMD fetus as harboring a *DMD* mutation and vice versa (see online Supplemental Fig. 6 and online Supplemental Table 7). Thus, targeting the whole *DMD* region with a tiling design is preferable to ensure the reliable detection of a recombination event within the *DMD* region. Third, because the *DMD* region is hemizygous for male probands, all phased heterozygous SNVs are informative for dosage imbalance analysis provided that the recombination event is checked and corrected for before analysis.

Considering the abovementioned gene-specific factors, we postulate that our approach targeting the whole *DMD* region with tiling design provide the best approach for noninvasive prenatal diagnosis of DMD. Instead of using multiple haplotype blocks for repeated relative haplotype dosage analysis *(5, 11)*, we hypothesized that the whole *DMD* gene could be analyzed as 1 large haplotype block, and we compared directly the allele fractions of 2 phased maternal heterozygous alleles. Although the study reported by New et al., in which multiple haplotype blocks were used, demonstrated the clinical applicability of that design to an array of autosomal recessive disorders *(11)*, we believe that the current approach using 1 large haplotype block is a simpler and more straightforward method, at least for DMD. Although the fetal genotypes from all 4 DMD families were accurately predicted, the application of the current approach to routine molecular testing may have several limitations, in particular regarding noninvasive prenatal diagnosis. First, the current proband-based phasing approach may lead to misdiagnosis if separate recombination occurs both in a proband and in a new fetus. In addition, it was basically impossible to discern whether the recombination event observed in

the DMD-02 family had occurred in the proband or in the new fetus using the current proband-based phasing approach, although the inherited fetal genotype could still be correctly predicted. This disadvantage could be partly overcome by adding the grandfather to maternal haplotype phasing, as the maternal X haplotype inherited from the grandfather would theoretically be free of recombination. This approach of using the grandparents for phasing was introduced in a recent article by Meng et al. *(10)*. Second, as female patients or carriers of *DMD* mutations with no known proband have been increasingly identified, the current proband-based phasing approach also has a practical limitation in that setting. An alternative method that uses other family members for maternal haplotype phasing could be introduced to overcome this limitation, although it may still not be applicable to all at-risk couples without a proband. A grandfather or normal male child may be used for phasing via the same approach. Moreover, a carrier female or normal female child may also be used for phasing when the paternal genotype is available. Third, the optimal timing of testing and the least fetal DNA fraction required should be determined and validated in extended DMD families with various *DMD* mutations. In the current study, the earliest gestational time and the lowest fetal fraction that allowed successful noninvasive prenatal diagnosis were 6 weeks and 5 days and 5.8%, respectively. New et al. reported a successful case of prenatal diagnosis at 5 weeks and 6 days of gestation with a fetal fraction of 1.4% *(11)*. We believe that our results are compatible with those of the study performed by New et al. in terms of resolution, considering that 5–6 weeks of gestation might be the earliest period at which prenatal tests may be offered. However, data collection in extended families is needed, as the current study used only 4 families compared with the 14 families reported by New et al. Fourth, because the informative SNVs that are required for dosage imbalance analysis of maternally inherited alleles might be limited in number and located at greater distance, the current approach using 1 large haplotype block may be biased by false-recombination prediction. Thus, the extension of the applicability of the current approach to autosomal recessive disorders should be demonstrated separately.

Besides the technical considerations, there are complex ethical and socioeconomic issues to be addressed before such an approach can be implemented in the clinic. Currently, no curative therapy is available for DMD, although some therapeutic molecules are under clinical trial *(23)*. However, considering the current method is best fitted to clinical circumstances in which the presence of an affected proband is the reason for prenatal testing, noninvasive determination of fetal genotype in the early gestational weeks could provide an autonomy-based reproductive option to the parents.

Despite the need to overcome these various hurdles, our approach for the comprehensive genetic diagnosis of the proband and noninvasive prenatal diagnosis using a single massively parallel targeted sequencing platform may provide a practical model for implementation of next-generation sequencing technology to clinical genetics.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: J.H. Chae, grant from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute, funded by the Ministry of Health and Welfare, Republic of Korea (grant number: HI13C-1468-050013); and Seoul National University Hospital and SK Telecom grant (grant number: 800-20120447).
Expert Testimony: None declared.
Patents: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

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