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SPECIAL TOPIC ISSUE ON ADVANCES IN THE DIAGNOSIS OF SINGLE GENE DISORDERS

cfDNA screening and diagnosis of monogenic disorders – where are we heading?

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ABSTRACT

Cell-free fetal DNA analysis for non-invasive prenatal screening of fetal chromosomal aneuploidy has been widely adopted for clinical use. Fetal monogenic diseases have also been shown to be amenable to non-invasive detection by maternal plasma DNA analysis. A number of recent technological developments in this area has increased the level of clinical interest, particularly as one approach does not require customized reagents per mutation. The mutational status of the fetus can be assessed by determining which parental haplotype that fetus has inherited based on the detection of haplotype-associated SNP alleles in maternal plasma. Such relative haplotype dosage analysis requires the input of the parental haplotype information for interpretation of the fetal inheritance pattern from the maternal plasma DNA data. The parental haplotype information can be obtained by direct means, reducing the need to infer haplotypes using DNA from other family members. The technique also allows the assessment of complex mutations and has multiplexing capabilities where a number of genes and mutations can be assessed at the same time. These advantages allow non-invasive prenatal diagnosis of fetal monogenic diseases to be much more scalable. These applications may drive the next wave of clinical adoption of cell-free fetal DNA testing. © 2018 The Authors Prenatal Diagnosis Published by John Wiley & Sons Ltd

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BACKGROUND

Since the discovery of cell-free fetal DNA in maternal plasma,[1] the way to identify fetal genotype without risk of miscarriage has changed remarkably. Applications of non-invasive prenatal diagnosis (NIPD) methods have been adopted for clinical use and include the determination of fetal sex for sex-linked diseases, *RHD* status, and some monogenic diseases.[2,3] NIPD for monogenic diseases is mostly relevant for pregnancies with positive family history or abnormal sonographic findings.

There are two main analytical approaches for non-invasive diagnosis of autosomal recessive diseases and X-linked diseases, namely the relative mutation dosage (RMD) and relative haplotype dosage (RHDO) methods.[4–6] Both of these approaches allow for the determination of which maternal allele the fetus has inherited despite the presence of an excess of maternal DNA among the cell-free fetal DNA in maternal plasma. RMD is based on assessing the ratio of mutant and wild-type alleles present in maternal plasma, statistically determining the fetal genotype at high sensitivity and specificity.[5,7] While RMD can be implemented with relatively simple instrumentation,[7] it is most suited for the detection of a single targeted mutation in one test,[5,8] and requires the use of probes specific for the family mutation.[9] On the other hand, RHDO deduces the fetal inheritance by comparing the relative ratios of the parental haplotypes present in maternal plasma (Figure 1).[3] To perform RHDO analysis, parental DNA haplotyping information is needed. Haplotype refers to how genetic features, for example, mutations and polymorphic alleles, are physically associated on the same copy of a chromosome. The relative concentration between the two maternal haplotypes, spanning the genomic region of clinical interest, in maternal plasma is analyzed by RHDO, determining the fetal genotypes statistically.[4,6,10,11] RHDO can be applied across the whole genome to decipher the fetal genome noninvasively.[4] Yet, RHDO can be performed in a targeted manner based on targeted sequencing of genomic regions relevant for disease diagnosis.[6,10,11] The targeted approach may be the more cost-effective and ethically appropriate approach for clinical use. However, how much cheaper it is when compared with whole genome approach would be dependent on the number of sequenced reads that can cover the informative SNPs, the size of the target gene panels, read length, sequencing reagent types, and the price of the capture probes.

The fetal inheritance of the genes of interest are assessed indirectly by SNP linkage analysis. SNP linkage analysis obviates the use of mutation-specific probes. Multiple genes can be assessed in a single assay regardless of mutation

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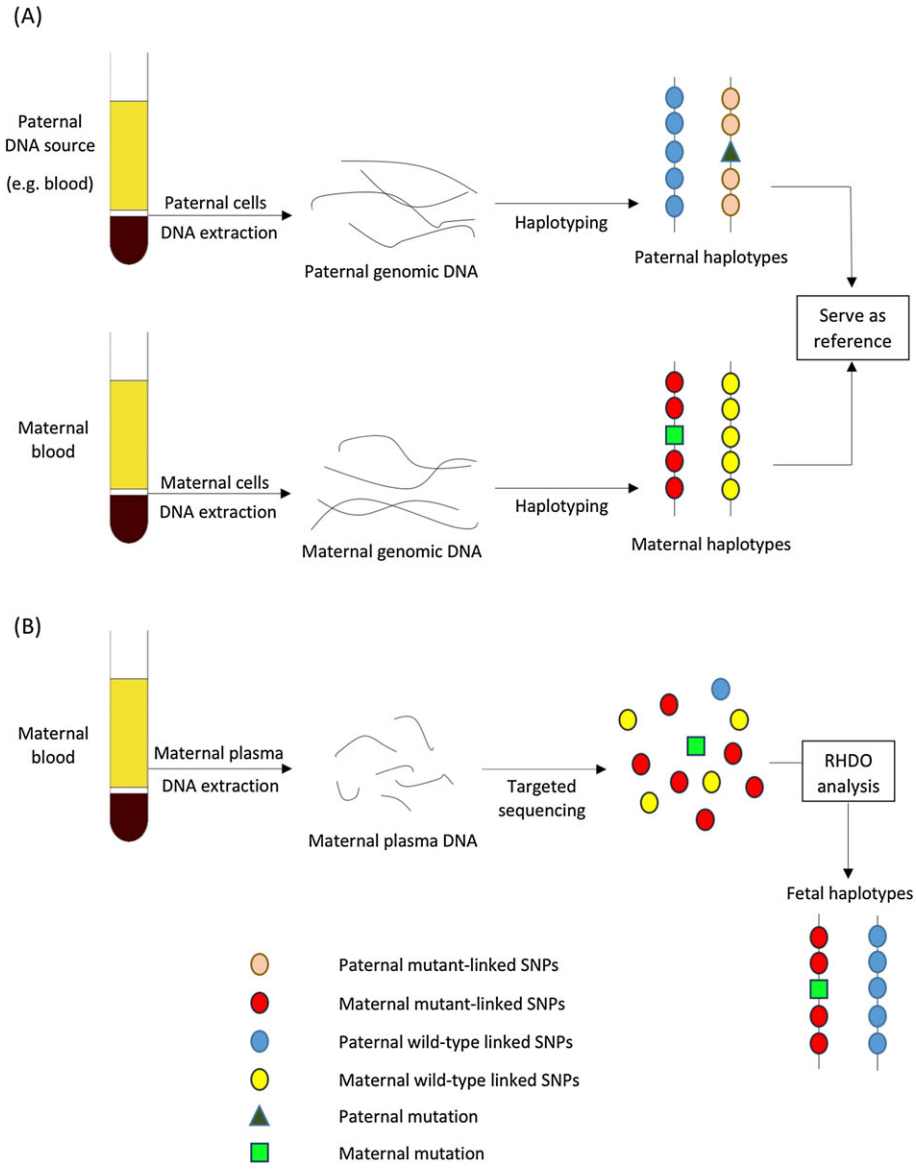

Figure 1 Schematic diagram for the general principle of non-invasive prenatal diagnosis of monogenic diseases. (A) Parental haplotypes are first identified. Maternal blood and paternal blood or other DNA sources are collected. Maternal and paternal cells undergo DNA extraction, yielding parental genomic DNA. Extracted genomic DNA is then sequenced and phased into haplotypes, where circles denote polymorphic alleles, triangle represents the paternal mutation, and square represents the maternal mutation. The haplotype results serve as the reference for further analysis. (B) To determine fetal genotypes, DNA from maternal plasma is extracted. The extracted plasma DNA undergoes targeted sequencing. The sequenced reads are aligned and analyzed by relative haplotype dosage (RHDO) with the reference to the parental haplotypes, to determine the fetal inheritance. In the example shown in this figure, the fetus inherits the mutation from mother and wild-type haplotype from father, indicating that it is a carrier

types.[6,12,13] However, parental DNA haplotyping has not been a trivial task, and has generally been based on inference techniques either by statistical means using population haplotype information or based on familial structure through the analysis of a proband's DNA.[4,6,10] However, proband DNA may not always be available, and occasionally, it may be difficult to delineate due to the inheritance of identical mutations from both the mother and father.[13]

A recent study published by Hui and colleagues[14] adopted a method that directly haplotyped the DNA of each parent, removing the reliance on the availability of proband DNA, resulting in a universal method that is applicable to the NIPD of various monogenic diseases. Here, we shall discuss the principles behind the method and how it achieves universality in disease detection, thus, broadening the clinical applicability of NIPD.

UNIVERSAL HAPLOTYPE-BASED NIPD

Methodological principles

A number of procedural steps are involved in non-invasive fetal genotype assessment by RHDO. Cell-free DNA including fetal DNA in maternal plasma is sequenced. Parental haplotypes are identified from maternal and paternal genomic

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DNA (Figure 1A). The relative abundance of each of the maternal and paternal haplotypes in maternal plasma are compared (Figure 1B).[14] From each parent, the fetus would have inherited the haplotype present at higher abundance in maternal plasma. Because plasma DNA is naturally fragmented into pieces of <200 BP long, haplotype information cannot be derived directly from the plasma DNA molecules. Instead, the parental haplotype information derived from genomic (cellular) DNA of the parents serves as a scaffold for assigning which haplotype any one maternal plasma DNA molecule belonged to. The approach can be used generally for genotyping the fetus across the whole genome or selected parts of the genome. To determine the disease status of the fetus with respect to any mutant alleles that it may inherit from its parents, haplotyping of the parental DNA would also involve determining which of the parental haplotypes is/are linked to the mutant alleles.

The approach employed by Hui and colleagues[14] employs a form of a direct haplotyping method, namely linked-read sequencing,[15] in which parental haplotypes were determined directly without a proband sample. To perform linked-read sequencing, genomic DNA of the parents needs to be extracted in a manner that maintains its high-molecular-weight nature (Figure 2). The long molecules of genomic DNA, in average longer than 50 kilobases, are then partitioned almost individually into emulsion with 10×™ barcoded gel beads. Each bead contains an abundance of the same set of oligonucleotide barcodes, but different beads have a different set. The barcoded oligonucleotide primers and reagents within the beads bind to random locations along the long DNA molecule, fragmenting it into short DNA fragments tagged with the same barcode in a particular gel bead (Figure 2). These barcoded short DNA fragments are then prepared to become libraries for sequencing. After sequencing, the short sequence reads that shared the same barcode as well as aligning to the same chromosomal region can be deemed as having originated from one long DNA molecule. The linking of the short DNA molecules enables the original haplotype to be resolved. After the assembly of a haplotype block has been completed by exhausting the short sequence reads with the same barcode, the size of the phased haplotype can be further extended by linking adjacent haplotype blocks that showed overlapping informative SNP alleles. In particular, haplotype blocks overlapping the mutation site are identified and phased accordingly, in which sequenced reads with the same barcode as the mutant-link reads are phased into the same haplotype, while reads linked with the wild type allele are phased into the opposite haplotype. The determined parental haplotypes serve as the reference for further analysis (Figure 1). The parental haplotype could cover the whole genome or only the genomic loci of clinical interest.

Relative haplotype dosage is then used to determine the fetal mutation status. To yield fetal-derived DNA, maternal plasma DNA is extracted and prepared into libraries. The plasma DNA library is enriched with target capture probes covering SNPs upstream and downstream of the desired disease gene loci for targeted sequencing. After sequencing, the short plasma DNA sequenced reads are mapped to the human genome. At the same time, by referring to the parental haplotypes, each maternal plasma DNA read is assigned to the maternal or paternal, mutant or normal haplotype. The number of DNA reads that are assigned to each haplotype is tabulated and included in the subsequent statistical analyses. To assess the fetal inheritance of the maternal mutation, the relative abundance of plasma DNA molecules aligning to each of the two maternal haplotypes are compared, namely the RHDO process. Hui et al.[14] based their statistical interpretation on the sequential probability ratio test.[16] The fractional fetal DNA concentration can be measured by either SNP-based approach or the ratio of *ZFY* and *ZFX* genes on chromosome Y and X, respectively. The determined fetal fraction is then taken into account in the calculations for further enhancement of the accuracy of the analysis. In the simplest implementation of this fetal genotyping approach, the paternal-specific alleles inherited by the fetus can be identified due to their presence in maternal plasma because such alleles are not present among the maternal genomic DNA. However, Hui et al.[14] used the Kolmogorov-Smirnow test[6] to statistically determine which paternal haplotype block was more abundant. This approach minimizes the chance of incorrect paternal allele classification resultant from sequencing errors at the polymorphic base. In addition, it allows the recombination sites between paternal haplotypes to be pinpointed at high precision.

Apart from linked-read sequencing, Vermeulen and colleagues[17] have employed targeted locus amplification (TLA) strategy for parental haplotype phasing. The TLA method exploits the nature of the spatial proximity of genomic loci on the linear chromosome in the nucleus.[18] It is performed by crosslinking sequences around the gene of interest, these are then digested to allow for *in situ* ligations. Ligation products containing an SNP of interest (or the pathogenic mutation) are then selectively amplified by inverse PCR and sequenced. Any variants detected within the same ligation product are assigned to the same haplotype, thus, phasing the parental alleles. The maternal plasma DNA also undergoes targeted sequencing. Fetal inheritance is then analyzed with RHDO.[17]

The TLA approach does not require any specialist equipment, making it amenable for implementation into a clinical genetic laboratory, whereas linked-read sequencing requires specific 10×™ Genomics instruments that may not be accessible to a clinical laboratory. On the other hand, the TLA approach requires additional laboratory procedures to phase the parental alleles for each family prior to sequencing the maternal plasma DNA. Nevertheless, both methods demonstrate that the combination of direct haplotyping and targeted maternal plasma DNA sequencing can provide robust NIPD for monogenic diseases.

Universal applicability

To offer NIPD of monogenic diseases, the combined use of direct haplotyping and RHDO analysis provides a generic solution both clinically and technically.[14,17] First, the specimens for testing can be readily obtained from couples in a non-invasive fashion. In the clinic, physicians only need to collect peripheral blood from the pregnant woman and blood or other source of genomic DNA from her partner for

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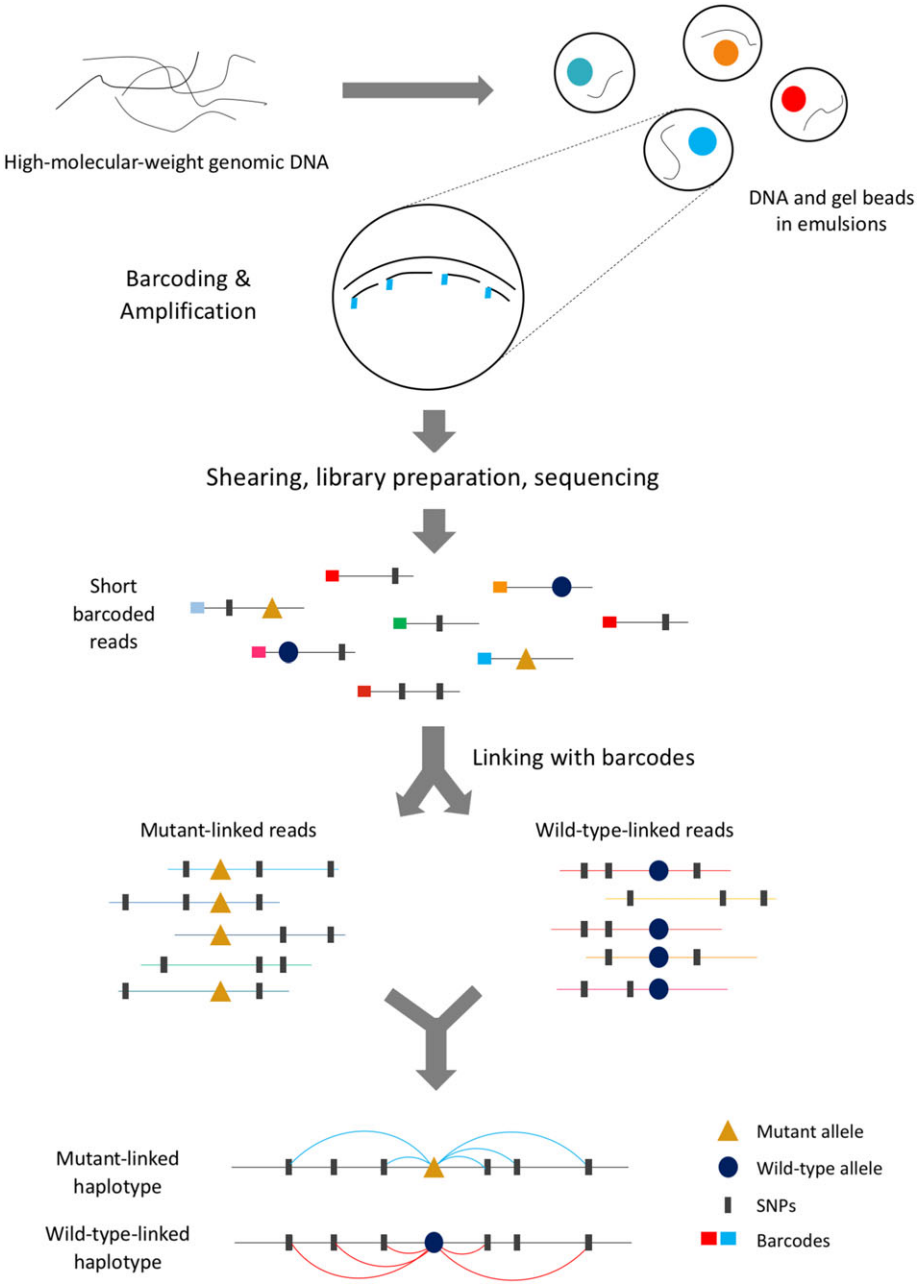

Figure 2 Schematic diagram for the process of linked-read sequencing. High-molecular-weight genomic DNA from parents, with barcoded gel beads, is distributed into separated emulsion partitions. Each gel bead contains millions of copies of barcoded primers, but different beads have a different set. Barcoded oligonucleotide primers and reagents bind along the genomic DNA randomly, resulting in numerous short DNA fragments tagged with the same barcode in a particular partition. Libraries are prepared using these barcoded short DNA fragments and sequenced. The sequenced short reads with the same barcode and aligned to the same chromosomal region can be deemed to have originated form the same long DNA molecule. By linking the short DNA molecules with the same barcode, haplotype blocks can be obtained. The size of phased haplotypes can be extended by further linking adjacent haplotype blocks with overlapping informative SNP alleles. Haplotype blocks overlapping the mutation site are identified and phased accordingly. Sequenced reads with the same barcode as the mutant-linked read are phased into the same haplotype, while those linked with the wild-type read are phased into the opposite haplotype

determining the parental haplotypes as well as for prenatal diagnosis of the fetal inheritance of monogenic disease of interest. Some previous protocols require DNA from a proband in addition to parental blood for haplotype inference in each family. The adoption of an efficient and accurate direct haplotyping method circumvents the need to collect samples from other family members for parental haplotype determination and thus require only samples from the parents. In comparing the cost of these methods, the direct haplotyping method requires the processing of fewer samples than the proband-required RHDO methods, thus, reducing the cost of library preparation and sequencing. However, this is offset by

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the requirement of 10×™ genome equipment and specialized reagents for the current form of the linked-read sequencing protocol, meaning that this method is more expensive. Nonetheless, the direct haplotyping approaches are particularly valuable in the situation where the proband's sample cannot be obtained. Furthermore, as the protocol is not restricted to the use of the 10× technology, it is hoped that there may be alternative direct haplotyping methodologies available in the future to render the approach more economical to implement.

Second, in contrast to the use of mutation-specific assays, which are tailor-made for each mutation, the application of massively parallel sequencing technology enables simultaneous screening of multiple gene loci for the causative sequence variants and informative SNPs (Table 1). For many genetic diseases, multiple mutations in the same gene or even mutations located on different genes could cause apparently similar pathologies. For example, any of more than 200 mutations in the *HBB* gene can lead to beta thalassemia.[19] It is time-consuming and costly to develop or perform numerous mutation-specific assays to target all possible mutations for different diseases. In fact, the mutation-specific assays might not have been developed in time for a relevant pregnancy when a couple presents for prenatal assessment. Because RHDO is based on interpreting the fetal inheritance using SNP alleles linked to the mutant allele or normal allele, target sequencing protocols that cover SNPs on gene loci of interest can be readily deployed as long as the carrier status of the parents are known. Although linkage analysis is universally applicable to many inherited mutations, it is not designed for detecting a new genetic variation that arises during gametogenesis or embryogenesis, also known as *de novo* mutation. *De novo* mutation-linked haplotype cannot be determined by analyzing the genetic information of parental somatic cells. Nevertheless, autosomal dominant *de novo* mutations can be examined by amplicon sequencing or deep sequencing of cell-free DNA.[12,20]

Moreover, haplotype-based cell-free fetal DNA analysis can expand the testing repertoire of NIPD. Because cell-free fetal DNA circulates in the maternal blood in highly fragmented form,[4] mutation-specific assay for some genomic loci (e.g. mutations in regions with similar sequence context) or mutation types (e.g. large structural variants) cannot be readily designed (Table 1). However, the inheritance of fetal mutations can be inferred by analysis of the linked polymorphisms. For example, the *CYP21A2* gene and its pseudogene shared >98% homology, which pose particular challenges for the design of mutation-specific assays. On the other hand, fetal genotypes can be inferred by linkage analysis through RHDO.[6] RHDO analysis has also been shown to facilitate the detection of fetal inheritance of common inversion mutations in *F8* gene without the need for precise determination of the inversion breakpoints in each patient.[10]

Relative haplotype dosage can be performed in a genome-wide manner or by targeting multiple genes. In short, we could analyze a panel of mutations in one or multiple genes as well as multiple patient samples with mutations on different genes in one test. Targeted sequencing assays or hybridization probes can be predesigned to capture one gene at a time or several clinically important monogenic disease loci in one panel as well as the highly heterozygous SNPs located upstream and downstream of the gene. Therefore, the same set of probes can scan a panel of mutations and detect adequate number of informative SNPs for different families. This can also reduce the turnaround time and cost for clinical service.

The development of one generic non-invasive prenatal testing protocol to detect a spectrum of mutations in one or multiple genes can facilitate the implementation of NIPD for monogenic diseases. Therefore, NIPD can be more readily applied to pregnant women whose fetus is at risk of different spectrum of monogenic diseases.

CLINICAL IMPLEMENTATION

With the development of a universally applicable method for NIPD of monogenic diseases, the spectrum of pregnancies whom might benefit from the test is widened. Here, we explain some clinical scenarios whereby the technology mentioned earlier might be applied.

Pregnancies with a known family history

If the carrier status of the couple is already known and mutations determined from prior investigations, parental haplotyping could be performed for the relevant genomic regions. The haplotype information could then be used for interpreting the maternal plasma DNA sequencing results to determine the fetal genotype for a current, as well as any future, pregnancy. If there is a known family history but the

Table 1 **Applications of RMD and RHDO for NIPD**

	RMD	RHDO
Clinical applications	• To detect SNPs and small deletions	• To detect SNPs, mutations including large structural variants, and mutations in regions with similar sequence context
	• With a predesigned assay, it is more cost-effective to detect single mutation with simple instrumentation	• More efficient to analyze a panel of mutations in one or multiple genes
	• Suitable for detecting common mutations in the population	• Can multiplex multiple patient samples with different mutations in one test
Limitations	• Cannot detect large structural variants and mutations in regions with similar sequence context	• Need to phase parental haplotypes
	• Need to design mutation-specific assay	• Not suitable for *de novo* mutations

RMD, relative mutation dosage; RHDO, relative haplotype dosage; NIPD, non-invasive prenatal diagnosis.

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carrier status of the parents is not known, the mutation identification and haplotyping of the parents can be performed in one step. During the linked-read sequencing procedure for haplotyping, the targeted sequencing can be designed to provide adequate coverage of the gene loci in search for mutations. When the parents are confirmed to be carriers and that the fetus is at risk, one may then proceed to the maternal cell-free DNA sequencing to determine the fetal genotype.

General screening

The universal haplotype-based NIPD test can be combined with population screening programs for carrier identification or prenatal screening of certain monogenic diseases. The American College of Obstetricians and Gynecologist has recommended pre-pregnancy or prenatal carrier testing for some relatively common diseases, such as spinal muscular atrophy, cystic fibrosis, and hemoglobinopathies.[21] The profile of diseases for carrier screening can be tailored according to family history of individual pregnancies or the ethnicity.[22] On the other hand, some centers now provide expanded carrier screening that involves the screening of a large panel of clinically important monogenic diseases.[22,23] As mentioned in the scenario earlier, if carrier screening is performed independently, parental haplotyping can be performed subsequent to any positive findings. The carrier status and linked haplotypes can be documented in the patient's health record and then retrieved when NIPD by maternal plasma DNA sequencing is required during pregnancy.

Alternatively, the carrier identification process and the intention to perform NIPD can be combined as one step. Targeted sequencing can be performed in a manner to identify carrier mutations as well as the linked polymorphic alleles at the same time. The haplotyping algorithm can be combined with optimized variant search parameters based on existing pathogenic mutation database. If mutations are identified among the parental DNA, one could then proceed to the fetal genotyping part of the test by sequencing the maternal plasma portion. This approach can be offered to couples with family history of monogenic diseases or when monogenic diseases are suspected during routine prenatal ultrasound scanning or maternal blood examination.

RESULT INTERPRETATION

The statistical algorithms adopted for the interpretation of the cell-free fetal DNA NIPD tests, RMD, and RHDO,[4,8] have been designed to independently evaluate both the likelihood that the fetus has inherited the mutant allele and the likelihood that the fetus has inherited the wild-type allele. The algorithm has also incorporated stringent filtering criteria, which considers the chances of sequencing errors, bias due to hybridization and mapping efficiency, and fetal fraction, to minimize incorrect classification. If the statistical evidence is not strong enough to support the classification of either the fetus having inherited the mutant allele or wild-type allele, the algorithm would most likely return an 'inconclusive' result instead of committing on an incorrect classification of the fetal mutational status. Existing data showed that cell-free DNA-based prenatal test identifies the fetal genotype with a high degree of accuracy.[6,10,12,14,17,24–26] Such stringent disease classification thresholds have been applied because the performance of non-invasive prenatal testing for monogenic diseases should aim for a definitive diagnostic level because most pregnancies tested are at high risk, often between 25% and 50% chance of an affected fetus.

For the implementation of the cell-free DNA-based test as a diagnostic test for prenatal determination of fetal inheritance of monogenic disorders to be successful, some factors should be considered. One of the foremost consideration is the validation of clinical sensitivity, specificity, and predictive value of the approach by large-scale studies on more pregnancies. There are multiple factors that should be considered when an inconclusive result is obtained, such as low fetal fraction, low total plasma DNA amount, multiple pregnancies, and the presence of a vanishing twin, maternal somatic mosaicism, or maternal history of organ transplantation.[25,26] If a recombination event occurs at a genomic region near the mutation, there is a chance of incorrect fetal genotype classification if the resolution to pinpoint the recombination is not high enough, which is dependent on an interplay of the distribution of the informative SNPs, the fetal fraction and sequencing depth. Therefore, the recombination rate of the target genes should also be considered when implementing these approaches.

IN THE FUTURE

The universal protocol can expand the scope for cell-free DNA-based NIPD of monogenic diseases, especially for those monogenic mutations with no prior screening protocol. Although a predesigned mutation-specific assay can efficiently detect or exclude the fetal inheritance of some common mutations in the community, such as ΔF508 pathogenic variant in *CFTR* gene that leads to cystic fibrosis,[24] for many mutations that are relatively rare and variable, it is not efficient to design mutation-specific assay for each family. Therefore, it is preferable to offer one generic haplotype-based protocol so more families can access NIPD to identify the fetal inheritance of monogenic mutations.

It is also important to consider the economic implications of implementing the test. To offer the test at an affordable price, we have demonstrated that it was possible to determine fetal inheritance without genotyping any affected relatives. Furthermore, we can optimize the experimental protocol for multiplex and targeted sequencing to minimize the reagents and sequencing power required. For example, we can design probes that target the gene of interest and highly heterozygous SNPs adjacent to the genes of interest for efficient informative SNP identification as well as haplotype construction. Moreover, because genomic analysis will generate large amount of data, the cost for data processing and storage should also be considered.

The introduction of a streamlined protocol for carrier identification and prenatal diagnosis can alleviate the time constraint for analysis and thus provide more time for the parents for any decision.

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CONCLUSION

Noninvasive fetal genotyping is becoming more easily accessible. In the past, NIPD was mostly for the assessment of paternally inherited mutations because such mutations are readily distinguishable from the maternal DNA sequences in maternal plasma. Due to the high background level of maternal DNA in plasma, RMD and RHDO methods were developed to determine the maternal inheritance of the fetus. RMD assays had to be tailor-made for each family. RHDO was initially performed with the use of proband's DNA information. Now that direct haplotyping methods can be incorporated with RHDO analysis of maternal plasma DNA, the clinical implementation of NIPD can become more streamlined. The universal haplotype-based NIPD method provides a way that one protocol alone can be used for most mutations of a particular disease, in which no mutation-specific assay is needed. NIPD services could therefore become more scalable with increased throughput and efficiency. However, as with any prenatal tests, professional bodies and the community need to determine which diseases are suitable candidates for prenatal assessment. Cost–benefit comparisons between potentially widened scope of NIPD versus the more conventional targeted, and often invasive, prenatal diagnostic approach for pregnancies at high risk for monogenic diseases need to be conducted to guide service development in this area.

WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- NIPD for some monogenic diseases is now in clinical service.
- Current NIPD is based on bespoke mutation-specific assays, or haplotype inference which requires proband DNA.

WHAT DOES THIS STUDY ADD?

- The application of universal haplotype-based NIPD can be extended to most pregnancies at risk for monogenic diseases.
- The universal protocol circumvents the need for bespoke assays or access to proband DNA.

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