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SYMPOSIUM: FUTURES IN REPRODUCTION
REVIEW

Non-invasive prenatal testing using massively parallel sequencing of maternal plasma DNA: from molecular karyotyping to fetal whole-genome sequencing

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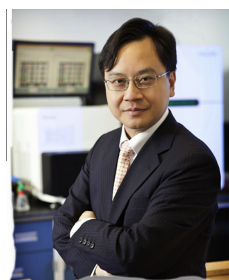
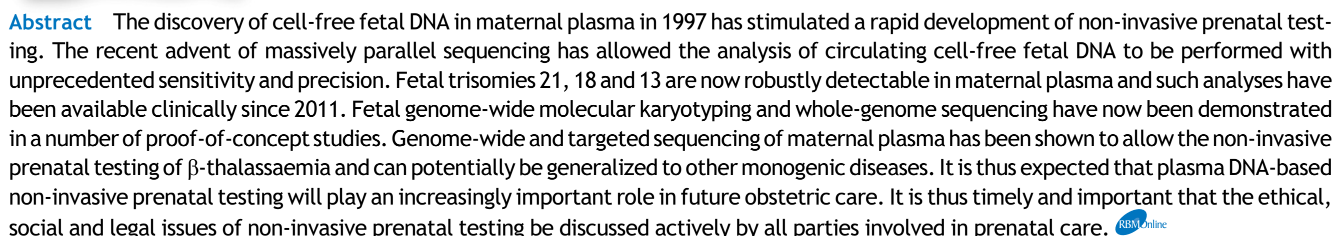


Dennis Lo is the Director of the Li Ka Shing Institute of Health Sciences and the Chairman of the Department of Chemical Pathology of The Chinese University of Hong Kong. In 1997, Dennis Lo and his co-workers reported the presence of cell-free fetal DNA in the plasma of pregnant women. Since then, he and his co-workers have elucidated the fundamental biological characteristics regarding circulating fetal DNA as well as its clinical applications in non-invasive prenatal testing. In recognition of his work, Dennis Lo has won numerous awards, including a 2005 State Natural Science Award from the State Council of China and the Ernesto Illy Trieste Science Prize from the Academy of Sciences for the Developing World. He was elected to the Royal Society in 2011 and as a Foreign Associate of the US National Academy of Sciences in 2013.



Abstract The discovery of cell-free fetal DNA in maternal plasma in 1997 has stimulated a rapid development of non-invasive prenatal testing. The recent advent of massively parallel sequencing has allowed the analysis of circulating cell-free fetal DNA to be performed with unprecedented sensitivity and precision. Fetal trisomies 21, 18 and 13 are now robustly detectable in maternal plasma and such analyses have been available clinically since 2011. Fetal genome-wide molecular karyotyping and whole-genome sequencing have now been demonstrated in a number of proof-of-concept studies. Genome-wide and targeted sequencing of maternal plasma has been shown to allow the non-invasive prenatal testing of β-thalassaemia and can potentially be generalized to other monogenic diseases. It is thus expected that plasma DNA-based non-invasive prenatal testing will play an increasingly important role in future obstetric care. It is thus timely and important that the ethical, social and legal issues of non-invasive prenatal testing be discussed actively by all parties involved in prenatal care.

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KEYWORDS: cell-free fetal DNA, circulating nucleic acids, Down syndrome screening, fetal DNA in maternal plasma, next-generation DNA sequencing, non-invasive prenatal testing

Introduction

The discovery of cell-free fetal DNA in maternal plasma in 1997 has opened up new possibilities for non-invasive prenatal testing (NIPT) (Lo et al., 1997). Circulating fetal DNA exists in maternal plasma at a mean fractional concentration of approximately 15% (Chiu et al., 2011). Fetal DNA is cleared rapidly from maternal plasma following delivery

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and thus there is no risk of fetal DNA persistence from one pregnancy into the next one (Lo et al., 1999). These characteristics have made circulating fetal DNA an attractive platform for developing NIPT. Indeed, early work had focused on the detection of fetal sex, for the prenatal investigation of sex-linked genetic diseases (Costa et al., 2002) and congenital adrenal hyperplasia (Rijnders et al., 2001) and for the detection of paternally inherited sequences which are absent in the mother's genome: for example, for fetal *RHD* genotyping (Faas et al., 1998; Finning et al., 2008; Lo et al., 1998) and for detecting paternally inherited gene mutations (Chiu et al., 2002).

With the advent of massively parallel sequencing (MPS), the sensitivity and precision with which one can analyse circulating fetal DNA for NIPT have been greatly enhanced. This review attempts to summarise a number of recent developments in this area.

Detection of fetal chromosomal aneuploidies

If a pregnant woman is carrying a fetus with trisomy 21, then the fetus would release an increased amount of chromosome 21-derived DNA sequences into maternal plasma, when compared with the other chromosomes. Thus, provided that one could measure the relative contributions of DNA from different chromosomes in maternal plasma precisely, then one should be able to detect the presence of a trisomic fetus non-invasively. In 2007, Lo et al. (2007) first reported single-molecule counting as one approach through which such precise measurement could be achieved. For such an approach, the higher the number of molecules that are counted, the higher is the analytical precision. Furthermore, the precision that one would need to achieve to detect fetal trisomy is related to the fractional fetal DNA concentration. Hence, the lower is the fractional fetal DNA concentration in a particular maternal plasma sample, the higher is the number of molecules that one would need to count to achieve the detection of the trisomic fetus. This concept was first demonstrated using digital PCR (Lo et al., 2007), a process whereby PCR is carried out on extremely diluted samples containing either one or no target molecules and many amplification reactions are carried out simultaneously. Then, the number of positive reactions is counted, which should provide a measure of the number of target molecules in the original sample.

With the advent of MPS (Schuster, 2008), an even more powerful method for counting millions or even billions of DNA molecules has become available. Compared with digital PCR, the use of MPS allows the DNA contained within a particular maternal plasma sample to be used much more efficiently. Thus, for digital PCR, the DNA molecules that can be counted are those containing the primer-binding sites. The remaining DNA molecules within the sample, which do not contain the primer-binding sites, will be 'wasted', even if they are derived from the chromosome of interest (e.g. chromosome 21). MPS, on the other hand, would allow virtually any DNA molecule contained within the plasma sample to be sequenced and to contribute towards the counting process (Chiu et al., 2009). Indeed, multiple studies have shown that such theoretical predictions are indeed correct and that MPS would allow fetal trisomy 21 to be detected robustly from maternal plasma (Chiu et al., 2008, 2011; Ehrich et al., 2011; Palomaki et al., 2011; Sehnert et al., 2011). It has been demonstrated that the precision of using MPS to measure the representation of chromosomes 13 and 18 is less than that for chromosome 21 (Chiu et al., 2008), possibly as a result of the so-called 'GC bias' (Guanine/Cytosine), as most MPS sequencers in common use require the use of an amplification step prior to performance of the actual sequencing. Nonetheless, through the use of appropriate bioinformatics algorithms that correct for such bias, it has been shown that trisomies 13 and 18 can also be detected with high sensitivity and specificity from maternal plasma (Bianchi et al., 2012; Chen et al., 2011; Dan et al., 2012; Lau et al., 2012). However, the current data suggest that, even with such corrections, the testing accuracies for trisomies 13 and 18 are still inferior to that for trisomy 21, thus indicating that either the GC bias has not been corrected completely by the current algorithms and/or that there are additional factors that have yet to be optimized. There are also recent data suggesting that the use of a single-molecule sequencer, which does not require a prior amplification step, might be beneficial for the detection of trisomy 18, although the application of such a platform for detecting trisomy 13 would require additional optimization (van den Oever et al., 2013).

The MPS process used in the above-mentioned publications is based on the random or shotgun sequencing of DNA molecules in maternal plasma (Chen et al., 2011; Chiu et al., 2008, 2011; Ehrich et al., 2011; Lau et al., 2011; Palomaki et al., 2011; Sehnert et al., 2011; Bianchi et al., 2012; van den Oever et al., 2013). As such random sequencing protocols would analyse sequences from across the whole-genome, including sequences which are of clinical interest (e.g. chromosome 21 sequences) as well as those that might not be clinically significant for a particular application, several groups have argued that it might be more cost effective to use targeted sequencing protocols. Such targeted sequencing protocols have been described and successfully used for detecting a number of chromosomal aneuploidies, focusing on targets on the chromosomes at risk of aneuploidies which are either non-polymorphic (Ashoor et al., 2012; Nicolaides et al., 2012; Norton et al., 2012; Sparks et al., 2012a,b) or those that are polymorphic (Liao et al., 2012; Zimmermann et al., 2012).

MPS-based methods for the NIPT of fetal chromosomal aneuploidies have been available clinically since 2011 in a number of countries, including those in North America, Asia and Europe. The American College of Obstetricians and Gynecologists has recently issued an opinion recommending the use of such testing for screening fetal aneuploidies in high-risk women (Committee Opinion, 2012). A similar opinion has also been published by the Society of Obstetricians and Gynaecologists of Canada (Langlois et al., 2013). It is anticipated that guidelines will be forthcoming in other countries.

Apart from abnormalities involving the entire chromosome, MPS-based analysis of maternal plasma DNA has also been shown to be useful for detecting Down syndrome caused by Robertsonian translocation (Lun et al., 2011), as well as microdeletions and microduplications (Jensen et al., 2012; Peters et al., 2011; Srinivasan et al., 2013; Yu et al., 2013). The latter two publications are particularly interesting because they show that a non-invasive molecular

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