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Clinical applications of high-throughput genetic diagnosis in inherited retinal dystrophies: Present challenges and future directions

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niques has greatly simplified the molecular diagnosis and gene identification in very rare and highly heterogeneous Mendelian disorders. Over the last two years, these approaches, especially whole exome sequencing (WES), alone or combined with homozygosity mapping and linkage analysis, have proved to be successful in the identification of more than 25 new causative retinal dystrophy genes. NGS-approaches have also identified a wealth of new mutations in previously reported genes and have provided more comprehensive information concerning the landscape of genotype-phenotype correlations and the genetic complexity/diversity of human control populations. Although whole genome sequencing is far more informative than WES, the functional meaning of the genetic variants identified by the latter can be more easily interpreted, and final diagnosis of inherited retinal dystrophies is extremely successful, reaching 80%, particularly for recessive cases. Even considering the present limitations of WES, the reductions in costs and time, the continual technical improvements, the implementation of refined bioinformatic tools and the unbiased comprehensive genetic information it provides, make WES a very promising diagnostic tool for routine clinical and genetic diagnosis in the future.

Key words: Next generation sequencing; Identification of novel causative genes; Inherited retinal dystrophies; Genetic diagnosis; Whole exome sequencing

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Core tip: This review focuses on the application of next generation sequencing (NGS)-based methods [whole genome sequencing, whole exome sequencing (WES), targeted exome sequencing] for genetic diagnosis and novel gene identification in hereditary retinal dystrophies. Advances over the last two years concerning NGS accuracy, reliability, development of bioinformatics tools, together with the drop in costs and time required for the

Abstract

The advent of next generation sequencing (NGS) tech-

analysis have allowed thirty novel genes to be identified, plus a large number of new mutations in previously reported genes. NGS techniques (particularly WES) are revolutionizing genetic diagnosis and have clear applications in clinical practice, helping to pave the way for personalized medicine. Present challenges and future directions are also discussed.

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INTRODUCTION

Inherited retinal dystrophies (IRDs) consist of a group of highly heterogeneous disorders at the genetic and clinical level. Until recently, the ever increasing number of causative genes (more than 200 so far) and mutations (more than 5000) (<https://sph.uth.edu/retnet/>) posed an enormous challenge for molecular diagnosis and limited the effectiveness of conventional mutational screening. However, the advent of next generation sequencing (NGS) technologies has completely revolutionized genetic diagnosis^[1,2]. Since the first application of exome sequencing using NGS to identify the causative gene in a very rare autosomal recessive disorder^[3], more than 150 new Mendelian disease genes have been reported using similar approaches^[4]. Focusing on IRD genes, NGS approaches [whole exome sequencing (WES), or whole genome sequencing (WGS)] have rapidly identified new causative genes, increasing the success rate of molecular diagnosis from 40% to almost 80%, depending on the number of cases analysed and the informativity of the family^[5-7]. It is foreseeable that NGS-based methods will be the technique of choice for future routine DNA diagnosis in IRDs and similar heterogeneous Mendelian disorders, since accuracy and efficiency increase while costs and time requirements drop continually^[8,9].

NGS-BASED DIAGNOSIS

The challenge posed by the molecular diagnosis of heterogeneous disorders prompted researchers to devise novel conceptual and technical approaches to help clinicians classify diseases, inform patients and families, and offer genetic counselling and prenatal diagnosis. The approaches they devised also provide the basis for a more efficient molecular-based therapy. Since the draft of the human genome was published, several high-throughput techniques have been devised. In the field of IRDs, commercially available microarrays for direct mutational screening (<http://www.asperbio.com/asperophthalmics>), customized resequencing microarrays

(restricted to several large diagnostic centres/units)^[10] and whole genome or targeted gene *SNP* genotyping arrays for linkage analysis (cosegregation and homozygosity studies) have paved the way either for mutation screening in reported known genes, or for the highlighting of new loci for candidate causal genes^[11]. Diagnostic efficiency ranged from 15%-44% in direct mutation screening microarrays-depending on the pathogenic allelic frequencies in the population, to 30%-70% for resequencing microarrays-depending on the number of genes included and the sequence quality^[12]. Indeed, direct analysis of known mutations and genes requires constant updating, and even so, many mutations remain undetected because they are private^[13]. Moreover, SNP genotyping for homozygosity mapping and cosegregation analysis has become a very informative genetic tool in many cases^[14,15].

WES EFFICIENCY IN THE DIAGNOSIS OF MENDELIAN DISORDERS

A survey of the IRD (syndromic and non-syndromic) genes identified in the last two years (up to 29) showed that all the approaches used to identify them involved NGS. The success of NGS as a diagnostic tool is due to: (1) the power of an unbiased genome scale analysis; (2) the increasing number of databases containing information on SNP allelic frequencies in different populations, which allows rare presumptive mutations to be discriminated from frequent genetic variants; (3) the relative simplicity of the currently standardized protocols; (4) powerful bioinformatics analysis; and (5) the fact that the data gathered is useful on its own.

Nonetheless, additional genetic information is still instrumental to increase the yield of molecular diagnosis since, despite the power of WES, gene identification in recessive IRDs (24) is far more successful than it is in dominant cases (5) (Table 1). The difference in this outcome is to be expected, since finding the relevant causative mutation in heterozygosis amidst the great number of genetic variants identified by WES (more than 20.000 on average) is not a straight-forward endeavour^[16]. In contrast, the requirement of a double heterozygous mutation (or even homozygosis) in the same gene for recessive cases, greatly diminishes the noise associated with such massive collection of data, and thus the number of putative causative genes, after the data has been filtered. While WES alone has pinpointed the causative gene in around 30% of the recessive IRD cases (years 2013-2014), adRD causative genes have proved to be more difficult to identify and require a combination of genetic approaches, such as linkage analysis, deletion mapping and targeted capture of candidates, to eventually single out the pathogenic mutation in a novel gene (10%)^[17]. The informativity of these genetic approaches has also greatly favoured gene identification in recessive cases (60%)^[9] (Table 1).

Table 1 List of retinal dystrophy causative and candidate genes identified in 2013-2014 and the strategy of identification

Gene	Retinal phenotype	Methodological approach
<i>ABCD5</i>	Recessive CRD, spastic paraparesis, white matter disease	Homozygosity mapping combined with WES ^[25]
<i>ADAMTS18</i>	arRD early onset	Homozygosity mapping combined with WES ^[43]
<i>ARLBP2</i>	arRP	Homozygosity mapping combined with WES ^[44]
<i>BBIP1</i>	arBBS	WES ^[45]
<i>C12orf65</i>	Recessive optic atrophy, spastic paraplegia and neuropathy	Linkage mapping WES ^[46,47]
<i>C21orf2</i>	Recessive CRD	Homozygosity mapping combined with WES ^[25]
<i>CSPP1</i>	Recessive JS	WES ^[48-50]
<i>DHX38</i>	arRP (early onset with macular coloboma)	Homozygosity mapping combined with candidate gene approach ^[51]
<i>DTHD1</i>	Recessive LCA, myopathy	Homozygosity mapping combined with WES ^[25]
<i>EMC1</i>	arRP	Homozygosity mapping combined with WES ^[25]
<i>GDF6</i>	arRD	Candidate gene sequencing ^[52]
<i>GPR125</i>	arRP	Homozygosity mapping combined with WES ^[25]
<i>HK1</i>	adRP, nonspherocytic hemolytic anemia, and neuropathy	Linkage mapping and WES ^[53]
<i>IFT27</i>	arBBS	Homozygosity mapping combined with candidate gene approach ^[42]
<i>IMPG1</i>	Dominant MD	Linkage mapping
	Recessive MD	WES and candidate gene sequencing ^[54-56]
<i>ITM2B</i>	Dominant RD, dementia	WES combined with linkage mapping ^[57]
<i>KIAA1549</i>	arRP	Homozygosity mapping combined with WES ^[25]
<i>KIZ</i>	arRP, arCRD	WES ^[58]
<i>LRIT3</i>	arCSNB	WES ^[59]
<i>MVK</i>	arRP, recessive mevalonic aciduria	WES ^[60]
<i>NEK2</i>	arRP	WGS ^[6]
<i>NR2F1</i>	Dominant optic atrophy, intellectual disability	Deletion mapping
		WES and deletion mapping ^[61,62]
<i>PCYT1A</i>	arCRD with skeletal disease	WES and targeted candidate gene sequencing ^[63,64]
<i>POC1B</i>	Recessive CRD	WES ^[65]
<i>PRPF4</i>	adRP	Targeted capture NGS ^[41]
<i>RAB28</i>	arCRD	Homozygosity mapping combined with WES ^[66]
<i>RDH11</i>	arRP	WES ^[67]
<i>SLC7A14</i>		WES ^[68]
<i>TUB</i>	arRD with obesity	Homozygosity mapping combined with WES ^[69,70]
<i>TTL5</i>	Recessive cone and CRD	WES ^[71]

ar: Autosomal recessive; ad: Autosomal dominant; CRD: Cone-rod dystrophy; RD: Retinal dystrophy; RP: Retinitis pigmentosa; BBS: Bardet-Biedl syndrome; JS: Joubert syndrome; LCA: Leber congenital amaurosis; MD: Macular dystrophy; CSNB: Congenital stationary night blindness; WES: Whole exome sequencing; WGS: Whole genome sequencing; NGS: Next generation sequencing.

WGS VS WES VS TARGETED EXOME SEQUENCING IN ROUTINE DIAGNOSIS

At present, many groups rely on NGS-based techniques for genetic diagnosis of IRDs (and other Mendelian disorders)^[18]; WES is the most common of these approaches (Table 1). Nonetheless, a few attempts using WGS or targeted exome sequencing have also been reported. In the latter, long polymerase chain reaction amplifiers spanning the exons of reported *RP* genes^[17] or lately, customized exome capture of the coding exons of a selected set of genes, have been developed with a wide range of diagnosis success (40%-80%)^[19-22]. Customized approaches allow different degrees of refinement and are dependent on the optimization of the techniques and the prioritization of the type of mutations to be identified. For instance, if copy number variants (CNVs) are suspected, the coverage and high quality of the reads constitute one focus of improvements^[23]. Nonetheless, the cost of customized capture arrays for a list of causative disease genes is still much higher than that of conventional capture arrays for WES, and the genetic information provided is limited to the candidates analysed. Mutations in non-selected or previously unreported genes will

remain undetected. WES is becoming the most popular choice, particularly since the reliability of the technique and the quality of the analysis software have greatly increased (though there is still room for improvement), and microRNAs and transcript untranslated regions are also included in some exon capture array versions^[24]. Overall, the reported success rate for IRD diagnosis in randomly selected familiar and simplex cases account for 74%-80% of the mutation pool in some studies^[25].

WGS for the molecular diagnosis of retinal dystrophy (RD) has been attempted with moderate success (56% of molecular diagnosis and the identification of a new causative gene)^[6]. The main reason behind this massive genome sequencing approach was to analyse coding and noncoding regions in order to detect structural and copy number variants and to evaluate highly polymorphic SNPs. Although the WGS reported in this work facilitated the detection of two structural pathogenic variants (which would probably have escaped detection with WES), the fact that no pathogenic mutation in the large noncoding fraction of the genome was identified, and that 7 out of 16 patients remained undiagnosed after the considerable effort required to screen the whole genome, pose some questions about the suitability of WGS in routine RD genetic diagnosis.

Table 2 Possible genetic cause in undiagnosed patients after whole exome sequencing

Genetic variants	Technical restrains	Alternative approaches
MicroRNAs and lncRNAs	Not sequenced	Inclusion in the capture
Deep intronic	Not sequenced	RNASeq
		WGS
		Targeted re-sequencing
Variants in regulatory regions	Not sequenced	WGS
		Targeted re-sequencing
Large deletions	Mostly undetected	Detectable in homozygosis
		In heterozygosis can be detected in comparison with controls (if high coverage)
		WGS
		Targeted re-seq
CNVs	Mostly undetected	High coverage
		WGS
		Targeted re-seq
		CGH
Pathogenic trinucleotide repeats	Short reads not covering the whole expansion	Triple repeat based PCR
Structural chromosomal variants	Undetectable	FISH
		WGS
		Targeted Long PCR coupled to NGS
Aneuploidies	Undetectable	Conventional cytogenetics FISH
		WGS

lncRNAs: Long non-coding RNAs; CNVs: Copy number variants; RNASeq: RNA sequencing; WGS: Whole genome sequencing; CGH: Comparative genome hybridization; PCR: Polymerase chain reaction; FISH: Fluorescent *in situ* hybridization; NGS: Next generation sequencing.

PRESENT LIMITATIONS OF WES IN GENETIC DIAGNOSIS

Although NGS-based methodologies allow comprehensive genomic analysis on an unprecedented scale, none of them is free from technical constraints. The conventional WES diagnostic strategy is based on exon capture by nucleic acid hybridization. Even though continuous improvements to the method have continually been implemented (capture optimization, and higher coverage and sequencing accuracy), not all the pathogenic mutations can be detected^[26]. One main issue that needs to be addressed without delay is the implementation of unified bioinformatics tools for accurate mapping and reliable variant-calling software, particularly for small indels (insertions/deletions) and CNVs^[8,27]. Other pending issues include the detection of mutations in genomic regions that escape the capture methods currently available, such as small exons, regulatory regions, deep intronic variants and chromosomal structural variations that do not affect exons (inversions and deletions) (Table 2).

When the main focus is basic research and the analysis is restricted to a small genomic region highlighted by linkage or homozygosity, custom targeted genome re-sequencing is a viable alternative to WES^[22,28]. However, for daily routine diagnosis, standard WES offers an appealing compromise between cost, time, comprehensiveness of data processing and efficiency.

UNEXPECTEDLY HIGH NUMBER OF IRD RECESSIVE PATHOGENIC VARIANTS IN THE CONTROL POPULATION

Knowledge of the underlying genetic structure of

human populations provides very valuable clues to help successfully identify pathogenic genes^[25,29], particularly in highly consanguineous cohorts where homozygosity by descent is suspected. Current data indicates that this assumption should be extended even in the absence of a positive family history, where both parents may be heterozygous for the same pathogenic allele. Not only may the unsuspected homozygosity of pathogenic alleles reveal a founder effect-which is informative in itself- but it is also one of the most useful genetic assumptions that can lead to the identification of novel causative alleles after WES^[25,30].

Notably, the wealth of genome information gathered by WES suggests that control individuals carry 10-20 pathogenic recessive mutations causative of Mendelian disorders^[3]. RD stands out as one of the most highly genetically heterogeneous monogenic disorders, and when we focus on the IRD causative genes-even when only null alleles are considered-22% of the control population (1 in 4-5 individuals) is heterozygous for at least one pathogenic mutation^[31]. This high prevalence is still an underestimate because missense and splicing mutations have not been included, and neither have all the IRD genes been identified, which overall would probably account for 1 in 2 control individuals carrying a pathogenic recessive RD mutation. Such a high frequency of unaffected carriers has an important impact on genetic diagnosis since: (1) consanguinity would increase the risk of blindness in the offspring; (2) the comparison of a newly identified genetic variant with control individuals in databases to assess pathogenicity could be misleading; and (3) many patients would by chance bear an additional pathogenic allele besides the causative mutations, which would hamper the molecular diagnosis. This last point would lead to

Table 3 List of prioritized candidates according to the clinical phenotype or X-linked pattern of inheritance

Main candidate gene	Disease
CNGB3, CNGA3	Achromatopsia
RHO	adRP
VMD2	Best disease
CYP4V2	Bietti crystalline dystrophy
RDS/PRPH2	Central areolar choroidal dystrophy
CHM	Choroideremia
LRP5, FZD4, TSAPN12	Familial exudative vitreoretinopathy
RDH5, RLBP1	Fundus albipunctatus
NR2E3	Goldman-Favre-Enhanced S-cone syndrome
CEP290	LCA
MFRP	Nanophthalmia
NDP	Norrie disease
SAG	Oguchi disease
RS1	Retinoschisis
RECQL4	Rothmund-Thompson syndrome
ABCA4, RDS/PRPH2	Stargardt disease
USH2A	Usher syndrome
VCN	Wagner syndrome
RPGR	XLCD, XLCRD
RPGR, RP2	XLRP, RP simplex

adRP: Autosomal dominant retinitis pigmentosa; LCA: Leber congenital amaurosis; XLCD: X-linked cone dystrophy; XLCRD: X-linked cone-rod dystrophy; XLRP: X-linked retinitis pigmentosa.

false assumptions of dominant effects of recessive alleles, and explain compound heterozygosity in some consanguineous pedigrees, and open the can of worms of digenic inheritance^[31]. In addition, reports of the synergic addition of pathogenic alleles in families with several phenotypes are now emerging, which would seem to call for a new conceptual molecular framework for genotype/phenotype correlations.

Another issue revealed by WES when trios (two parental samples in addition to the patient sample) are analysed is the unexpectedly high frequency of *de novo* mutations, which strengthens the case for reconsidering dominance along side recessivity in simplex cases^[22].

PENETRANCE AND EXPRESSIVITY REVISITED: MODIFIER GENES AND WES

Incomplete penetrance and variable expressivity are two genetic phenomena frequently associated with human disease, mainly due to additional genetic factors influencing the final phenotype. From the molecular point of view, genes and proteins interacting and/or regulating the function of the causative gene exert a modifying effect, which could enhance or diminish the pathological outcome in patients bearing the same causative mutation. Identifying the modifier genes has been, and still is, one of the most important challenges in clinical and genetic diagnosis. WES is instrumental in unveiling modifier alleles by direct comparison of the DNA sequences of affected members of the same family, frequently displaying different phenotype severity^[6,28,32].

As there is a continual increase in WES-generated

data on genetic variants, the pool of modifier genes likewise grows and diagnostic inferences will become more accurate, thus providing the grounds for a more precise prognosis.

EMPOWERING GENETIC DIAGNOSIS OF IRDS BY WES: CANDIDATE PRIORITIZATION CRITERIA, GENETIC INFORMATION AND INTERACTION NETWORKS

So far, NGS-based approaches have mostly been considered for the identification of causative genes in very rare Mendelian disorders when the gene is unknown or mutation screening involves a large number of genes and exons, as is the case of highly heterogeneous diseases. However, after progressive and substantial methodological refining, WES and other NGS-based techniques have leapt from bench to bedside, and are now feasible and attractive alternatives for routine diagnosis. They allow for comprehensive genomic screening, are increasingly affordable and robust, and last but not least, the bioinformatics analysis is becoming more accurate and user-friendly (even though a common standard framework for downstream variant mapping and calling analysis is still lacking)^[18,33].

Monogenic disorders caused by mutations in a major gene also will benefit from WES (NGS)-based diagnosis. The costs of Sanger sequencing of a large gene (e.g., *ABCA4*, *CEP290*, etc.) are no less than those of full exome sequencing (WES), but the benefits from the comprehensive information gleaned *via* the latter technique are far superior. To mention just a few: minor causative genes are included in the analysis, additional disease causing alleles in modifier genes will be also detected-and so their impact in the population genetic reservoir can be assessed; the molecular basis of rare clinical entities with ambiguous diagnosis can be identified; genotype-phenotype correlations will be more precisely defined; and genetic data on the patient drug response (pharmacogenetics) will be included. Indeed, the analysis of NGS-based data should be prioritized for the genes and variants that are most prevalent for a particular IRD and pattern of inheritance (for instance, in X-linked disorders) (Table 3). If no pathogenic variants are identified, the list of candidates should be expanded following prioritization criteria that include less frequent causative candidates for the same (or similar) phenotype, and finally, all the variants detected by WES under all possible assumptions of Mendelian inheritance should be considered^[22,34]. This is particularly relevant in simplex cases and pedigrees with a small number of patients, where dominant *de novo*, X-linked or very rare recessive mutations should be carefully considered. In this context, exhaustive human gene mutation repositories will be extremely informative tools to perform a rapid screening of reported mutations

and thus, simplify the genomic analysis^[35].

Indeed, intersection with previous or parallel genetic analysis has been and still is instrumental in pinpointing pathogenic alleles. For instance, SNP genotyping microarrays (6K Illumina) for linkage or homozygosity studies (Table 1 and references therein), or SNP-based cosegregation chips^[12] highlight the genetic loci where the gene/mutation identification efforts should be focused. This greatly simplifies matters and provides statistical support for the final molecular diagnosis. In fact, only one third of the novel *IRD* genes identified by NGS over the last two years (Table 1) were discovered without resorting to candidate prioritization using genetic data.

TAKING ON THE FUTURE: PARTS LIST, MAP, DIAGNOSIS, THERAPY

How many novel *IRD* causative genes remain to be identified? Based on the latest NGS results where all new genes explain either rare syndromic disorders with an accompanying *IRD* phenotype or cases with private mutations affecting very few patients, it seems very unlikely that any novel gene will account for a substantial fraction of unassigned cases^[6]. As most technical approaches do not cover the whole panoply of causative mutations, a percentage of mutations in already reported genes might have been overlooked. In fact, transcriptome analysis of healthy human retinas revealed more than one hundred previously unannotated genes, almost 30000 unreported exons (around a 3% increase) and over 20000 3' and 5' alternative splicing sites^[36]. This unprecedented transcript diversity is a serious challenge for mutation identification, as these regions are not yet included in commercial exome enrichment kits and RNASeq of patient neural tissues is not feasible. Thus, optimization of molecular diagnosis in *IRD* demands, on the one hand, technical improvements for easy implementation and accuracy, and on the other, the widening of the genomic regions to include novel genes, exons and other regions of interest.

The great wealth of data gathered by conventional as well as high-throughput approaches demands a framework based on systems biology^[37]. To this end, unveiling the genetic networks underlying *IRDs*, although still fragmentary, is a valid approach. Ongoing efforts to integrate interactomes of photoreceptors^[38-40] are beginning to show the first promising candidates^[41,42]. Further work will allow the translation of this genetic information to the cellular and tissular contexts. Only a comprehensive view of the retinal pathways in health and disease can pave the way for effective therapies.

Finally, although not the main aim of this review, we should not overlook that any genetic laboratory working on WES and WGS data should abide to strict ethical guidelines that concern incidental findings relevant to the patient's health status but unrelated to the focus of the genetic testing.

CONCLUSION

To sum up, the generalized implementation of NGS-based analysis will foster more reliable genotype/phenotype correlations and provide a more holistic view of the genetic factors that cause and modify the severity of the phenotype. Even though 100% diagnosis will not be reached soon and there are new challenges and questions to address, the comprehensive genetic data gathered by NGS will definitely help the clinicians and patients in securing diagnosis, improving prognosis and recommending therapy. It is foreseeable that in the near future, clinical management of the patient will become more personalized and thus more effective.

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Comparison of next generation sequencing-based and methylated DNA immunoprecipitation-based approaches for fetal aneuploidy non-invasive prenatal testing

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Conflict-of-interest: Elisavet A Papageorgiou is currently employed by and owns shares of NIPD Genetics. Philippos C Patsalis also owns shares of NIPD Genetics. Elisavet A Papageorgiou and Philippos C Patsalis have filed a PCT patent application for the MeDIP real time qPCR based NIPD approach (PCT Patent Application No.PCT/1B2011/O00217). Voula Velissariou and Georgia Christopoulou declare that they have no conflict of interest.

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Abstract

Over the past few years, many researchers have attempted to develop non-invasive prenatal testing methods in order to investigate the genetic status of the fetus. The aim is to avoid invasive procedures such as chorionic villus and amniotic fluid sampling, which result in a significant risk for pregnancy loss. The discovery of cell free fetal DNA circulating in the maternal blood has great potential for the development of non-invasive prenatal testing (NIPT) methodologies. Such strategies have been successfully applied for the determination of the fetal rhesus status and inherited monogenic disease but the field of fetal aneuploidy investigation seems to be more challenging. The main reason for this is that the maternal cell free DNA in the mother's plasma is far more abundant, and because it is identical to half of the corresponding fetal DNA. Approaches developed are mainly based on next generation sequencing (NGS) technologies and epigenetic genetic modifications, such as fetal-maternal DNA differential methylation. At present, genetic services for non-invasive fetal aneuploidy detection are offered using NGS-based approaches but, for reasons that are presented herein, they still serve as screening tests which are not readily accessed by the majority of couples. Here we discuss the limitations of both strategies for NIPT and the future potential of the methods developed.

Key words: Next generation sequencing; Differential methylation; Epigenetics; Fetal aneuploidy; Methylation dependent immunoprecipitation; Non-invasive prenatal testing

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Core tip: Non-invasive prenatal screening and diagnosis of fetal aneuploidies has been a challenging field

for many researchers. Different methodologies have been developed, mainly based on next-generation sequencing and epigenetic modifications. At present, non-invasive prenatal testing services are offered using next generation sequencing-based technologies which have great potential, but currently they present with certain limitations. Epigenetic approaches may overcome some of these limitations and seem to have promising potential for wider applications.

Christopoulou G, Papageorgiou EA, Patsalis PC, Velissariou V. Comparison of next generation sequencing-based and methylated DNA immunoprecipitation-based approaches for fetal aneuploidy non-invasive prenatal testing. *World J Med Genet* 2015; 5(2): 23-27 Available from: URL: <http://www.wjgnet.com/2220-3184/full/v5/i2/23.htm> DOI: <http://dx.doi.org/10.5496/wjmg.v5.i2.23>

INTRODUCTION

Invasive procedures such as chorionic villus sampling (CVS) and amniocentesis are a prerequisite for the prenatal diagnosis of fetal chromosomal abnormalities, either by conventional and/or molecular fetal karyotyping, or other molecular cytogenetic methods. Although these approaches yield accurate results, the rate of pregnancy loss attributed to CVS or amniocentesis is estimated to be 1.0% to 2.0%^[1]. This considerable procedure-related risk of pregnancy loss has motivated researchers to try to develop non-invasive approaches in order to provide safer healthcare service.

Since the discovery that fetal cells circulate in the maternal blood during pregnancy^[2], numerous researchers worldwide have put great effort towards exploring the possibility of non-invasive prenatal investigation of the fetal genetic constitution. Initially, the focus of investigation was on circulating fetal nucleated cells, where detection of fetal gender and aneuploidies was made possible, mainly by applying FISH subsequent to cell sorting^[3-5]. Even though preliminary results were promising, the development of a commercially available application has failed to date, mainly due to certain inherent limitations of the method. Firstly, the rarity of fetal cells in the maternal circulation made it very difficult to isolate a satisfactory number for investigation^[4-6]. Secondly, the poor quality of the isolated fetal cells made the application of FISH on the nuclei problematic, resulting in less reliable results. Most importantly, the observation that fetal cells may remain in the maternal circulation for several years after their release, presents a serious problem for non-invasive prenatal investigation of subsequent pregnancies^[7,8]. Nevertheless, researchers have not given up this approach entirely and attempts are still being made to overcome limitations^[9-13].

The discovery of cell free fetal DNA (cffDNA) in maternal plasma during pregnancy by Lo *et al.*^[14] in 1997, gave rise to a whole new opportunity in the field of

non-invasive prenatal testing (NIPT). Its origin is proven to be either trophoblastic or from embryonic cells in the maternal blood which have undergone apoptosis^[15]. It has also been demonstrated that cffDNA is cleared from maternal plasma within a few hours after delivery^[16], making its study specific to the current pregnancy. Although cffDNA is detectable from the early stages of pregnancy^[17] and increases during its progression^[18,19], it is demonstrated to account only for 3.0% to 6.0%^[14,20] of total free DNA in maternal plasma. A more recent study utilizing microfluidics, re-estimated the cffDNA fraction to a median of 9.7% in the first trimester^[21,22]. The relatively small amount of fetal DNA in maternal plasma presents one of the most serious technical challenges for whichever technology is implemented for investigation. Furthermore, the fact that fetal DNA is 50% identical with that of the mother makes the attempts for fetal aneuploidy testing even more challenging.

CURRENTLY APPLIED METHODS

During recent years independent teams from all over the world have focused on developing methods for NIPT using cffDNA, mainly testing for fetal aneuploidy^[23]. Despite applying different strategies including SNP and allelic ratio analyses, none have managed to produce a widely available test, mainly because they depend on informative genotypes or fetal gender^[24-26]. On the other hand, next-generation sequencing (NGS) technologies have made great progress in the field, resulting in commercially available NIPT services. In recent years, the use of commercially available tests for NIPT for trisomy 13, 18, 21 and sex chromosome aneuploidies has been introduced into routine antenatal care. Massively parallel direct sequencing reads from a tested chromosome are compared to others with the aid of sophisticated bioinformatics software, resulting in a relative chromosome dose. NGS-based methods are polymorphism independent and have the ability to detect aneuploidies. In a recently published meta-analysis of clinical validation and implementation studies the pooled weighted detection rate for trisomy 21 is reported to be > 99% and the false positive rate to be < 0.01%^[27]. Commercially available tests based on NGS technologies have been validated on large numbers of cases and have a very high sensitivity and specificity^[28-34] as well (Table 1).

Another promising prospective in NIPT is provided by methylated DNA immunoprecipitation-based (MeDIP-based) approaches. The discovery of fetal-maternal differentially methylated regions (DMRs)^[35] has facilitated the development of NIPT strategies by combining MeDIP with other downstream applications. Using the "epigenetic approach", a NIPT method based on MeDIP combined with quantitative polymerase chain reaction which proved to be of high precision in a proof of principle (100% sensitivity, 100% specificity)^[36] and larger validation study^[37] (100% sensitivity, 99.2%

Table 1 Validation and verification comparison of the most widely used commercially available non-invasive prenatal testing for trisomy 21

Company	Sequenome	Verinata (Illumina)	Ariosia	Natera
Test	"Materni T21 PLUS"	"Verify"	"Harmony"	"Panorama"
Sensitivity	99.6%-99% (209/212)	> 99% (90/90)	100% (81/81)	> 99% (25/25)
Specificity	99.8% (1468/1471)	99.8% (409/410)	99.97% (2887/2888)	> 99% (242/242)
False positive	0.2% (3/1471)	0.2% (1/410)	0.03% (1/2888)	0
No result rate	3.4%	5.8%	4.7%-5.7%	5.4%

specificity) was developed.

ADVANTAGES AND DISADVANTAGES

Approaches based on NGS are extremely powerful. Besides detecting whole chromosome aneuploidy, they have the potential to detect smaller chromosomal imbalances allowing for microdeletion/microduplication syndrome NIPT^[38]. However, although NIPT is already commercially available for the detection of a certain number of microdeletion/microduplication syndromes, further validation studies are needed^[39]. Taking into account the vast amount of data that NGS is capable of producing, it could be potentially be combined with other methodologies to generate non-invasive fetal whole genome sequencing^[40]. As impressive as this may seem at present, it is quite possible that this will materialize in the near future.

The impressive developments of NGS technologies are accompanied however by certain drawbacks. One important limitation is the low level of fetal DNA which is available for testing. This is overcome in MeDIP-based technologies which are based on fetal DNA enrichment, which then increases sensitivity substantially. However, MeDIP by which cfDNA hypermethylated regions are selectively enriched is a stage wherein bias may be introduced, influencing the test results. Therefore, it is very important to carefully select DMRs, optimize this stage and evaluate the overall performance allowing for this. Another drawback of NGS-based approaches is that the equipment/technology required is still not available in all clinical settings, making the service feasible only in large centers, such as those in the United States and China. Furthermore, the requirements for significant infrastructure, complex laboratory procedures, highly trained personnel and challenging bioinformatics analyses make NGS-based technologies costly and complex. In contrast, the "epigenetic approach", uses equipment that is available in most genetic diagnostic laboratories offering established genetic services, it is considerably cheaper and simpler and therefore it may be applied potentially worldwide and offered to a broader population. However, current MeDIP-based approaches focus mainly on fetal trisomy 21 and at present have not yet demonstrated their ability to detect other fetal aneuploidies and submicroscopic aberrations that NGS-

based technologies have proven to be capable of detecting. Moreover, large validation studies and future clinical application feedback data are awaited in order to re-evaluate the advantages and disadvantages of MeDIP-based NIPT tests.

THE FUTURE OF FETAL ANEUPLOIDY NIPT

Both NGS and MeDIP-based approaches yield risk classification results at present. This means that a probability is given for each condition investigated, and depending on whether the pregnancy is assessed as being high risk or not, the couples are counseled to proceed with confirmatory invasive diagnostic testing, usually fetal karyotyping after CVS or amniocentesis. False positive results lead to unneeded invasive procedures posing an undesirable risk of pregnancy loss, while false negative results may lead to the birth of an abnormal child. There is an argument that false negative NIPT results for trisomy 18 or 13 are unlikely to result in the birth of an abnormal child because both syndromes are most likely to present with serious ultrasound findings during pregnancy. Conversely, cases with trisomy 21 (Down syndrome) may not have any indications throughout the pregnancy and consequently, NIPT false negative trisomy 21 fetuses are more likely to be born^[41]. Therefore, NIPT for trisomy 13, 18 and 21 should be considered as a screening test rather than a diagnostic test, which should be robust, rapid and cost efficient. We believe that MeDIP-based tests meet these requirements for the reasons already presented, and moreover have certain advantages compared to NGS-based methods and therefore show great potential for large scale public service access. At present, if treated as a replacement for current biochemical screening tests, the resulting risk could be combined with that derived from ultrasound markers such as nuchal translucency measurement and others. The combined NIPT-U/S risk for fetal aneuploidy may provide a safer screening strategy compared to that offered to most couples today^[42]. The future aim is to eventually avoid invasive procedures and develop NIPT (testing) into NIPD (diagnosis).

For any NIPT used caution is needed when it comes to

genetic counseling, in order to avoid misunderstandings concerning diagnosis. There is an ongoing debate on ethical and policy issues related to NIPT and the European Society of Human Genetics/American Society of Human Genetics invite the scientific community to contribute to setting future guidelines for NIPT^[43].

CONCLUSION

During recent years there have been enormous advances in the field of fetal aneuploidy NIPT. Relevant genetic services are offered by academic centers and commercial companies worldwide, but not all future parents have access to this service. Our team is working towards developing a commercially available MeDIP-based test, that will be relatively inexpensive and easy to apply and from which more people can benefit. Looking ahead, we predict that epigenetic based approaches in combination with genetic-based approaches and advanced technologies (digital PCR, NGS) will contribute to the development of NIPT for more subtle fetal genetic abnormalities^[44], such as point mutations, microdeletion/microduplication syndromes, *etc.*

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