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

Marfany G *et al*. NGS diagnosis in inherited retinal dystrophies

Gemma Marfany, Roser Gonzàlez-Duarte

**Gemma Marfany, Roser Gonzàlez-Duarte,**Departament de Genètica, Facultat de Biologia, Universitat de Barcelona, 08028 Barcelona, Spain

**Gemma Marfany, Roser Gonzàlez-Duarte,** CIBER, Instituto de Salud Carlos III, 08028 Barcelona, Spain

**Gemma Marfany, Roser Gonzàlez-Duarte,** IBUB, Institut de Biomedicina de la Universitat de Barcelona, 08028 Barcelona, Spain

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**Correspondence to: Roser Gonzàlez-Duarte, PhD,** Departament de Genètica, Facultat de Biologia, Universitat de Barcelona, Avinguda Diagonal 643, 08028 Barcelona, Spain. rgonzalez@ub.edu

**Telephone:** +34-93-4021034

**Fax:** +34-93-4034420

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**Abstract**

The advent of next generation sequencing (NGS) techniques 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:** Inherited retinal dystrophies; Genetic diagnosis; Next generation sequencing; Whole exome sequencing; Identification of novel causative genes

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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 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/asper-ophthalmics), 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).

**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 PCR amplimers 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 UTRs 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 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.

**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 constrains. 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 bionformatics 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 false assumptions of dominant effects of recessive alleles, and explain compound heterozygosis 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)[8,33].

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 (see 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 30.000 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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**Table 1 List of RD causative and candidate genes identified in 2013-2014 and the strategy of identification**

|  |  |  |
| --- | --- | --- |
| **Gene** | **Retinal phenotype** | **Methodological approach** |
| ABCD5 | Recessive CRD, spastic parapesis, white matter disease | Homozygosity mapping combined with WES[25] |
| ADAMTS18 | arRD early onset | Homozygosity mapping combined with WES[43] |
| ARLBP2 | arRP | Homozygosity mapping combined with WES[44] |
| BBIP1 | arBBS | WES[45] |
| C12orf65 | Recessive optic atrophy, spastic paraplegia and neuropathy | Linkage mapping  WES[46,47] |
| C21orf2 | Recessive CRD | Homozygosity mapping combined with WES[25] |
| CSPP1 | Recessive JS | WES[48–50] |
| DHX38 | arRP (early onset with macular coloboma) | Homozygosity mapping combined with candidate gene approach[51] |
| DTHD1 | Recessive LCA, myopathy | Homozygosity mapping combined with WES[25] |
| EMC1 | arRP | Homozygosity mapping combined with WES[25] |
| GDF6 | arRD | Candidate gene sequencing[52] |
| GPR125 | arRP | Homozygosity mapping combined with WES[25] |
| HK1 | adRP, nonspherocytic hemolytic anemia, and neuropathy | Linkage mapping and WES[53] |
| IFT27 | arBBS | Homozygosity mapping combined with candidate gene approach[42] |
| IMPG1 | Dominant MD  Recessive MD | Linkage mapping  WES and candidate gene sequencing[54–56] |
| ITM2B | Dominant RD, dementia | WES combined with linkage mapping[57] |
| KIAA1549 | arRP | Homozygosity mapping combined with WES[25] |
| KIZ | arRP, arCRD | WES[58] |
| LRIT3 | arCSNB | WES[59] |
| MVK | arRP  Recessive mevalonic aciduria | WES[60] |
| NEK2 | arRP | WGS[6] |
| NR2F1 | Dominant optic atrophy, intellectual disability | Deletion mapping  WES and deletion mapping[61,62] |
| PCYT1A | arCRD with skeletal disease | WES and targeted candidate gene sequencing[63,64] |
| POC1B | Recessive CRD | WES[65] |
| PRPF4 | adRP | Targeted capture NGS[41] |
| RAB28 | arCRD | Homozygosity mapping combined with WES[66] |
| RDH11 | arRP | WES[67] |
| SLC7A14 |  | WES[68] |
| TUB | arRD with obesity | Homozygosity mapping combined with WES[69,70] |
| TTLL5 | Recessive cone and CRD | WES[71] |

BBS: Bardet-Biedl syndrome; JS: Joubert syndrome; MD: Macular dystrophy; WES: Whole exome sequencing; CRD: Cone-rod dystrophy; CSNB: Complete congenital stationary night blindness; WGS: Whole genome sequencing; NGS: Next generation sequencing.

**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 |

WGS: Whole genome sequencing; CGH: Comparative genome hybridization; CNV: Copy number variants.

**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 |
| LRPO5, FZD4, TSAPN12 | Familiar 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 |