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Seeds of Science

Cilia Disorders in the Genomics Era: Historical Overview and Commentary on Ciliopathy Diagnostics

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Abstract

Introduction: Motile and sensory (primary) cilia are organelles that are found on the surface of almost all cells. Defects in cilia cause a number of multi-organ diseases known as ciliopathies, which have clinically heterogeneous symptoms. This heterogeneity makes diagnosing cilia disorders challenging and clinicians often rely on genetic sequencing to delineate ciliopathies from other diseases. However, there is not a consensus on which sequencing tools are most optimal for ciliopathy diagnosis and research.

Methods: Here I review the implications of next-generation sequencing tools for ciliopathy diagnostics. I describe landmark studies that showed ciliopathies as genetic conditions and transition to the advantages and challenges of using next-generation sequencing techniques. In particular, I compare studies that utilized targeted sequencing with those that used whole-exome and/or whole-genome sequencing.

Discussion: High throughput screens can identify novel cilia genes and show promise as a robust diagnostic tool in clinical settings. Moreover, I compare the effectiveness of whole-exome and whole-genome sequencing both for basic science research and clinical applications, arguing that whole-exome sequencing is a sufficient first pass in clinical settings. I also acknowledge that ciliopathies are associated with many, both significant and insignificant, genetic variants making interpreting next-generation sequencing data an ongoing challenge for scientists and clinicians.

Conclusion: This review demonstrates the increasing body of knowledge on cilia genomics and highlights that next-generation sequencing will be integral towards optimizing diagnostics for these heterogeneous and debilitating group of disorders.

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Introduction

Cilia are present on almost every cell type in the human body. (Ishikawa and Marshall 2011) They are microtubule-based cellular projections that extend from the plasma membrane. Despite their prevalence, for many years, these hair-like projections, particularly non-motile cilia, were thought to be vestigial structures with no important function. (Wheway, Nazlamova, and Hancock 2018) However, after years of scientific disregard, “the cilium has emerged as a key organelle in numerous physiological and developmental processes”. (Ishikawa and Marshall 2011)

Cilia are assembled on basal bodies, which are derived from centrioles, and have a microtubule-based axoneme. (Horani et al. 2016) Cilia can be either motile, as seen in ependymal cells that line brain vesicles, or immotile/sensory, as seen in the photoreceptor cells of the retina or the collecting ducts of the kidney. (Reiter and Leroux 2017; Vladar and Stearns 2007) Motile cilia enable cell movement and the movement of fluids across the surface of cells. Sensory (primary) cilia are specialized for signal transduction and act as ‘antennae’, sensing extracellular signals like growth factors, odorants, and developmental morphogens.

Research has revealed that defective primary and motile cilia can result in a number of human diseases, including retinal degeneration, polycystic kidney disease, and primary ciliary dyskinesia. (Hildebrandt, Benzing, and Katsanis 2011) Since cilia are ubiquitous, mutations in cilia-related genes can cause multi-organ disorders, which are called ciliopathies. (Hildebrandt, Benzing, and Katsanis 2011) Ciliopathies can be either autosomal dominant or recessive and diagnosing these disorders can be very challenging as clinical presentations are often very heterogeneous. (Braun and Hildebrandt 2017) This creates a clinical need to optimize genetics-based diagnostic tools for ciliopathies. Moreover, there is a knowledge gap in which genetic variations are causative for ciliopathies. Here, I review the implications of using next-generation sequencing tools to diagnose ciliopathies and to identify novel genetic variants that are associated with cilia defects. I will highlight the transition from landmark linkage analysis to high throughput screens, while also discussing the strengths of different approaches, which include targeted next-generation, whole-exome, and whole-genome sequencing.

Based on this synthesis, I argue that whole-exome sequencing is a sufficient first pass in clinical settings, while whole-genome sequencing is more informative for basic science research.

Discussion

i. *Early Linkage Mapping Studies Associated Some Disorders with Variants in Cilia Genes*

A landmark study in the field of cilia genetics was the identification of a candidate gene associated with monogenic polycystic kidney disease (PKD). (Reeders et al. 1985) PKD is a common and often lethal multi-organ disease with around 12.5 million people affected worldwide. (Chebib and Torres 2016) The authors of this influential paper identified four families that had PKD-like symptoms and relied on traditional genome mapping techniques to identify the location of a potential causative gene. They used genetic markers and looked for linkages with a potential disease allele. Ultimately, linkage analysis and lod score calculations allowed researchers to map the PKD-1 gene to the short arm of chromosome 16. However, at the time, researchers did not know that this gene was related to the primary cilium. The link was established about a decade later when *another research group found a functional homologue of PKD-1 in a model organism, C. elegans*. (Barr and Sternberg 1999) These authors generated PKD-1 knock-out organisms and saw that PKD-1 is part of the signaling cascade that is necessary for cilia formation. This was one of the first instances where a cilia-related gene was associated with a human disorder and undoubtedly generated a lot of interest in finding genes that encode for structural or functional cilia proteins.

With an increase in genome mapping experiments, another group of researchers looked for genetic associations for a disease that is characterized by retinal dystrophy, obesity, renal malformations, and learning disabilities. (Forsythe and Beales 2013) This disease is now coined Bardet-Biedl syndrome (BBS) and for many years its molecular basis remained elusive. This was mostly due to the fact that different researchers were mapping it to different loci, with at least six loci being associated with phenotypic BBS. (Leppert et al. 1994) Different research groups focused on different genetic loci and translated them to model organism experiments to see where the encoded protein

localized. The commonality was that these proteins localized to the base of the primary cilium, the basal body. (Katsanis et al. 2000; Mykytyn et al. 2002) We now know that the basal body is necessary for cilia formation and, in most cases cilia maintenance, in ciliated cells. (Magescas et al. 2021; Breslow and Holland 2019) The fact that defects in the primary cilium can cause multi-organ disorders further fueled interest in dissecting the molecular mechanisms of ciliopathies.

ii. *Sanger Sequencing to Diagnose Known Ciliopathies*

As scientists started to learn more about the genetic causes of ciliopathies, Sanger sequencing of exons in known cilia genes became the standard for clinical diagnostics. Researchers built on the aforementioned work on BBS, which in 2011 was associated with 15 cilia genes. In one study, researchers did Sanger Sequencing on 55 families with BBS. (Chen et al. 2011) Sequencing 142 exons showed that 84% of the patients had potentially pathogenic variations in one of the 12 cilia genes. The mutational analysis also revealed 21 novel mutations in these known genes, including 10 novel missense variations and 2 frameshift mutations. These results show the high genetic heterogeneity that can lead to BBS and ciliopathies at large. In addition, this heterogeneity can lead to the following two challenges. Firstly, it can make predictive genotyping challenging, meaning that it is difficult to know whether or not someone who does not show a ciliopathy phenotype, but has a variant in a ciliary gene, will develop clinical symptoms. Secondly, Sanger sequencing relies on previous knowledge of ciliopathy genes, but it is possible that known genes or exons of genes are not adequate to get a whole genetic picture of a ciliopathy patient. Given the large genetic heterogeneity, it is possible that exons that were not sequenced might have pathogenic variants or there might be mutations in other unidentified ciliary genes.

iii. *Targeted Next-Generation Sequencing*

These challenges associated with Sanger sequencing coincided with a period where next-generation sequencing tools were becoming more and more prevalent in research and clinical settings. (Koboldt et al. 2013) After linking many renal disorders to cilia perturbations, scientists aimed to identify the mutation profile of ciliary genes in

autosomal recessive PKD patients. Thus, targeted next-generation sequencing emerged as a cost-effective and rapid way to assess variants in cilia genes. An important study assessed allelic variants in 191 structural and functional genes of the primary cilium in tissue samples from 7 PKD patients. (Skalická et al. 2018) This was a significantly more high-throughput experiment compared to early studies that found one or two genes associated with familial PKD. Illumina sequencing revealed pathogenic variants in 39 genes encoding various structural components of the primary cilium. The most frequently mutated genes were those that encode centriolar and centrosomal proteins; other studies have also shown mutations in centriolar genes as the most common causes of ciliopathies. (Hildebrandt, Benzing, and Katsanis 2011) Since centrioles are essential for cilia formation it is likely that these patients do not form cilia and as such have severe disease phenotypes.

Interestingly, none of the samples showed mutations in intraflagellar transport (IFT) proteins, which are a group of proteins that carry essential protein cargo to the tip of the cilium. This highlights the genetic heterogeneity of PKD since the same phenotype can be traced back to an autosomal recessive mutation in an IFT protein in another study. (Qin, Rosenbaum, and Barr 2001) However, I believe that the lack of IFT proteins could also be due to the small sample size of this study (n=7 patients). Moreover, this also highlights a weakness of targeted sequencing. It is possible that IFT genes were not sequenced comprehensively, preventing the researchers from identifying causal variants in these highly redundant genes. Given a large number of studies that show IFT proteins as causal factors in renal ciliopathies, I believe that IFT proteins are important for proper cilia function and thus a genetic cause for ciliopathies. (Reiter and Leroux 2017) The authors of this study did not conduct extensive sequencing of all known IFT proteins. Overall, targeted next-generation sequencing has been key in highlighting genetic variations that lead to cilia defects, but can miss some pathogenic variants that are implicated in ciliopathies.

iv. *Whole Exome Sequencing is an Effective Diagnostic Tool and Integral for Identifying Novel Ciliopathy Genes*

As the cost of per nucleotide next-generation sequencing continued to decrease, researchers started to complement targeted studies with exome-wide approaches. Whole-exome sequencing (WES) yields a more comprehensive analysis of genetic variations that lead to ciliopathies. Moreover, WES can reveal novel genes that are important for cilia function, while still generating rapid and high-depth results. WES was used on six families with members that had ambiguous ciliopathy-like symptoms. (Castro-Sánchez et al. 2017) It is important to highlight that these patients had not received an official ciliopathy diagnosis. Despite this ambiguous clinical presentation, all the patients had mutations in BBS genes, which are a group of genes that encode for basal body proteins. This group of BBS proteins, sometimes referred to as the 'BBSome' transport essential signal receptor proteins to the cilium and thus defects in the 'BBSome' impair the cilium's ability to detect external signals. (Jin and Nachury 2009) BBSome defects result in many ciliopathy phenotypes, as mentioned previously. Moreover, WES revealed mutations in three other genes that were proposed to be "novel candidate cilia genes." The functions of these genes are unknown, but they are all differentially expressed in dividing cells, suggesting that they might play a role in centriole function, which is essential for ciliogenesis. Examples like this show that WES is a useful strategy to diagnose patients with unclear phenotypes, while also revealing novel genes that might be related to cilia biology.

WES can also allow clinicians to understand the underlying genetics of chronic kidney disorders, a common ciliopathy phenotype. Knowing that kidney failure is due to primary cilia defects can guide optimal treatment. In this pursuit, a research group did WES on around 100 patients that had chronic kidney disease before age 25 and found that 7 of them had mutations in cilia genes. (Mann et al. 2019) Interestingly, one patient with a ciliopathy variant did not have any known family history of renal disease, so the mode of transmission remains an open question. Regardless, WES is an informative tool for ciliopathy patients that are in need of kidney transplantation management.

Moving onto disorders associated with defects in motile cilia, we can see that a large number of studies have also relied on WES. An important study analyzed primary ciliary dyskinesia (PCD), a defect of motile cilia that leads to chronic respiratory symptoms. This disease has an autosomal recessive mode of inheritance and is a known ciliopathy. (Horani et al. 2016) Researchers utilized whole-genome sequencing on one family and WES on another family and saw that all PCD patients had a homozygous loss-of-function in a ciliary axoneme gene. (Onoufriadis et al. 2014) However, this was not the only gene associated with PCD. Another group identified nine related subjects with PCD from geographically dispersed Amish communities and performed WES of two affected individuals and their unaffected parents. (Horani et al. 2012) WES confirmed the previously known autosomal-recessive mode of inheritance and identified a missense mutation in the HEATR2 gene as the causal variant in these families. (Lucas et al. 2020) This gene is essential for the stability of the inner microtubule structure that allows for the movement of motile cilia. (King 2016) This study shows that WES can be an effective diagnostic tool for PCD and also reveals that mutations in various ciliary genes can be sufficient for PCD. This begs the question of do all mutations that are linked to PCD generate the same phenotype?

Next-generation sequencing has been key in linking changes in different ciliary genes to phenotype severity. As of 2020, more than 40 different genes have been reported that cause PCD. (Lucas et al. 2020) There have been attempts at linking certain genes to milder phenotypes, but I think it is important to exercise caution as these links are made based on small numbers of patients with variations in any one gene. An example is DNAH9 mutations, a protein that is essential for axoneme stability, being associated with a mild respiratory phenotype. (Postema et al. 2020) Overall, WES is shown to be effective at diagnosing ciliopathies associated with defects in motile and/or primary cilia.

v. *Is Whole-Genome Sequencing More Robust at Diagnosing Ciliopathies?*

A growing body of literature shows that WES is an effective way of diagnosing ciliopathies, and yet exome-sequencing does not take into account a very large portion

of the DNA of patients. This begs the question of would whole-genome sequencing be more robust? While there is no literature that directly addresses this question, an important point to note is that almost all known causative variants that lead to ciliopathies are in the coding regions of the genome. Several studies have used WGS in families that have ciliopathies and only identified variants in coding regions.

However, WGS can be more effective at diagnosing patients that have atypical symptoms. Many studies show that WGS is impactful in identifying novel developmental disorders, and ciliopathies should not be an exception. (Wright et al. 2015) While WES is more prevalent, WGS is a valuable tool when diagnosing patients that have a diverse spectrum of disease characteristics, particularly since it can reveal changes in non-coding regions of other genes that can be contributing to the ciliopathy-like phenotype. In one recent study, scientists did WGS on 4 children with suspected ciliopathies. (Strong et al. 2021) The authors identified that all 4 children had mutations in cilia genes, but two patients had additional pathogenic variants for non-coding regions that are associated with liver disease.

In addition, WGS is important at identifying structural changes and copy number defects. One study that conducted WGS in 11 different families found a tandem duplication of 3 exons of an IFT gene in 8 families, which all had uncharacterized ciliopathies. (Geoffroy et al. 2018) Importantly, this IFT protein, which is involved with carrying the building blocks of cilia to the tip of the axoneme, had been missed by whole-exome sequencing. This study shows that ciliopathies are not just caused by nucleotide-level variants or deletions and that WGS is instrumental in detecting structural rearrangements, including copy number changes. Moreover, other studies have suggested that whole-genome sequencing is more powerful than whole-exome sequencing at detecting exome variants. (Belkadi et al. 2015) Lastly, it is known that transcription-level regulation is an important part of ciliogenesis. (Choksi et al. 2014) There is some evidence that mutations in ciliogenesis regulators would lead to mild to severe ciliopathies. For instance, proteins that regulate the expression levels of two cilia genes were shown to be associated with Joubert syndrome. (Lee et al. 2012) Mutations in this regulatory gene were shown to give rise to an ambiguous ciliopathy-like

phenotype. Such genetic mechanisms will be revealed as WGS on ciliopathy patients get more prevalent.

Overall, WES has been very effective at identifying known and find novel genetic variants that lead to ciliopathies. It is likely that it is sufficient as a first-pass in clinical settings. However, I do not think that a targeted-gene panel would be robust enough to rule out a cilia defect for a patient that has ciliopathy-like symptoms. WES would be an important follow-up for patients that had a 'normal' gene panel sequencing. In addition, WGS can play an important role in research by tackling a knowledge gap in cilia biology: can ciliopathies be associated with variations in non-coding regions of the genome?

vi. *Challenges of Using Whole-Exome and Whole-Genome Sequencing as Diagnostics*

We have demonstrated that whole-exome and/or whole-genome sequencing are important venues for ciliopathy detection and the identification of novel genes that are implicated in such diseases. Yet there are challenges associated with using WES and/or WGS in clinic for diagnostic purposes. Firstly, cilia genes tend to be highly variable and lack mutation hot spots. Many variants generate wild-type cilia and thus it can make diagnosing clinically ambiguous patients challenging. Moreover, different families tend to have different variations in the same gene, making developing precise diagnostic tools a complex task. Secondly, some cilia genes have duplicated regions in other parts of the chromosome. Take the PKD1 gene, for example. Several studies have shown that the high sequence similarity between the pseudogenes and their parent genes can obscure the detection of the pathogenic mutation. (Ali et al. 2019; Eisenberger et al. 2015) An added challenge is the fact that pseudogenes for original ciliopathy-associated genes are located proximal to the original gene. (Ali et al. 2019)

Moreover, mutations in the same ciliary gene can give rise to different symptoms, making it challenging for physicians to initiate genetic sequencing or interpret the results. For instance, mutations in the CEP290, a centrosomal protein, can lead to retina phenotypes and/or kidney problems. (Baker and Beales 2009) It is possible that it

can cause other symptoms as well and if physicians are not aware of them it might be difficult for them to associate genetic variants with the phenotype. Since the boundaries that separate ciliopathy symptoms are very fluid wide-scale implementation can be a challenge.

Lastly, there are some medical ethics challenges associated with using WES in clinic. WES may identify mutations in genes unrelated to the studied disease, which has major implications for patients and their relatives. This would not be the case with targeted sequencing and yet we have talked about the limitations of targeted sequencing in identifying novel ciliopathies. In addition, we do not know what the implications of a novel variant will be. As mentioned previously, many variants of unknown significance tend to be unproblematic for patients. Do these patients benefit from knowing that they have this variant? It is worth noting that these problems are seen in the context of other genetic disorders and need to be tackled via a multi-disciplinary collaboration that includes genetic counselors, physicians, and ethics specialists.

vii. *Discussion of Future Directions for Ciliopathy Genomics*

The increase in genomics data continues to reveal new cilia genes that are causative for ciliopathies. However, we are still far from a comprehensive list of ciliopathy genes. Firstly, we are far from generating a complete genetic map of a wild-type cilium. Analysis of single-cell RNA-sequencing data and WGS will continue to reveal new genes that regulate cilia function. (Patir et al. 2020) Secondly, scientists will continue to discover novel genetic variants in known cilia genes that contribute to ciliopathies. Thirdly, while I highlighted some molecular mechanisms that lead to ciliopathies, the exact process through which many of these genes impact the cilium is unknown, making translating our findings to therapeutics an open question in the field.

Another open question in the field of ciliopathies is whether or not we can link mutations in specific genes to a phenotype. As we have seen through this review, similar variants can lead to drastically different phenotypes and this makes precision treatment challenging. This can be tackled with a meta-analysis and bioinformatics approach. If we can combine next-generation sequencing and phenotype data for

ciliopathy patients, we can generate a 'ciliopathy' databank that associates each gene and variant to a phenotype. As this potentially open-source databank grows, scientists can notice trends and make statistically significant genotype to phenotype interpretations.

Furthermore, most research has focused on ciliopathies as monogenic (autosomal dominant or recessive) disorders. It would be interesting to see if some patients have multiple mutations in different cilia genes. Due to the basal body's close relationship to centrioles, it is likely that multiple mutations lead to embryonic lethality, however this might not be the case with all individuals. There are model organisms that are viable and show severe defects after introducing mutations in multiple cilia genes. (Cornils et al. 2016) So, it would be clinically important to see if an increase in the number of cilia mutations correlates with more severe ciliopathy phenotypes.

Lastly, there has been an increasing interest in studying links between cilia defects and neurodevelopmental disorders. (Valente et al. 2014) In a recent pre-print, a large research group associated autism spectrum disorder, intellectual disabilities, and attention deficit disorders with de novo or inherited variants in ciliogenesis regulators. (Harris et al. 2020) The study established that, in their cohort of 36 people, deleterious variants in transcription factors that regulate ciliogenesis are important causes for the aforementioned disorders. So far, most research on ciliopathies has been conducted on inherited mutations, but this study paves the way for research that looks at the impact of de novo mutations in cilia biology.

Conclusion

Here, I reviewed the impact of next-generation sequencing techniques on our knowledge of cilia defects and ciliopathy diagnostics. Over the last two decades the field has evolved drastically: from using chromosome mapping to utilizing next-generation genome-wide sequencing, which has allowed scientists to identify novel genetic variants that cause ciliopathies. These high throughput screens have been critical in showing the genetic heterogeneity of ciliopathies, alongside highlighting that cilia defects can cause multi-organ level phenotypes. This synthesis revealed that mutations in genes that

encode basal body (BBSome, CEP290, PKD-1), IFT, and microtubule-based axoneme proteins (HEATR2, DNAH9) can be causative factors for ciliopathies, with no clear correlation with clinical symptoms. Importantly, I attempted to associate mutations in specific genes to clinical phenotypes, and similar to other reviews (Strong et al. 2021; Tobin and Beales 2009; Oud, Lamers, and Arts 2017) saw that mutations which disrupt similar cellular processes can lead to varying clinical phenotypes. Thus, I believe that the real clinical power of this data will come when we integrate results from different studies that looked at the genetics of ciliopathies. As we start generating large biobanks for ciliopathies, we will be able to make more accurate diagnostics based on whole-exome or whole-genome sequencing data. Perhaps with a robust understanding of the impact of specific genetic variants, we can start creating evidence-based treatments for unique ciliopathies. Tailoring treatment towards each patient's genetics and clinical phenotype will pave the way towards an era of precision medicine for ciliopathies.

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Gardener Comments

Michael Tran (PhD in molecular cellular biology):
The author's focus is mainly on the use of next-generation sequencing tools to identify genes involved and does a beautiful job reviewing the history of its use. However, I would like to also further highlight the use of model systems to identify cilia related genes and how they might synergize with the studies highlighted by the author. Molecular biology including biochemistry and electron microscopy in cilia models have identified various ciliary components. Work in these systems have also elucidated the function of many of these proteins. However, the shortcoming of these studies is that their role in actual ciliopathies is unknown. So further efforts in these model systems in combination with more targeted next-generation sequencing could thereby circumventing some of the ethical challenges of WES. A future where we have a more complete list of all cilia related proteins would marry wells with these new sequencing techniques to help create a list of ciliopathy genes.

Ahmad Ozair, MD:
This is a thoughtful and comprehensive review of the literature on diagnostics of primary ciliary disorders that strongly merits publication.

Joe R:
This is the best-written article I've seen so far, and an example of the correct use of jargon: technical terms are explained in context, and used exactly and only where necessary to concisely communicate a technical concept. I can't speak to the accuracy of the studies or conclusions, as this is not my field, but as an overview of the state of the art in ciliopathy diagnosis, the paper's logic seems sound.

Jack Arcalon:
I wonder if this general line of research could also apply to or inspire research in neuro-degenerative diseases.

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