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Dawn of ocular gene therapy: implications for molecular diagnosis in retinal disease

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Personalized medicine aims to utilize genomic information about patients to tailor treatment. Gene replacement therapy for rare genetic disorders is perhaps the most extreme form of personalized medicine, in that the patients' genome wholly determines their treatment regimen. Gene therapy for retinal disorders is poised to become a clinical reality. The eye is an optimal site for gene therapy due to the relative ease of precise vector delivery, immune system isolation, and availability for monitoring of any potential damage or side effects. Due to these advantages, clinical trials for gene therapy of retinal diseases are currently underway. A necessary precursor to such gene therapies is accurate molecular diagnosis of the mutation(s) underlying disease. In this review, we discuss the application of Next Generation Sequencing (NGS) to obtain such a diagnosis and identify disease causing genes, using retinal disorders as a case study. After reviewing ocular gene therapy, we discuss the application of NGS to the identification of novel Mendelian disease genes. We then compare current, array based mutation detection methods against next NGS-based methods in three retinal diseases: Leber's Congenital Amaurosis, Retinitis Pigmentosa, and Stargardt's disease. We conclude that next-generation sequencing based diagnosis offers several advantages over array based methods, including a higher rate of successful diagnosis and the ability to more deeply and efficiently assay a broad spectrum of mutations. However, the relative difficulty of interpreting sequence results and the development of standardized, reliable bioinformatic tools remain outstanding concerns. In this review, recent advances NGS based molecular diagnoses are discussed, as well as their implications for the development of personalized medicine.

next-generation sequencing (NGS), retinal disease, molecular diagnosis, Leber's Congenital Amarosis (LCA), Retinitus Pigmentosa (RP), Stargardt disease, APEX, personal medicine

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Personalized medicine is revolutionizing healthcare. Our impending ability to understand patient's defects on the molecular level, and to specifically tailor treatment on the same scale, may allow the development of therapies to treat previously incurable diseases and improve treatment across the board [1]. Inherited ocular diseases, in particular, are ideal targets for gene therapy, and have been the subject of numerous clinical trials ([2–8], reviewed in [9]) and studies [10–15] for a number of reasons. First, the clinical and genetic heterogeneity of ocular disorders makes it difficult to identify compounds that can treat all patients effectively, suggesting that tailored treatments may be necessary [16]. Second, a large number of genes underlying disease have been identified [17] that cumulatively account for a majority of cases [18–20], making it possible for accurate diagnosis at the molecular level. Third, gene therapy in the eye has been highly successful in both animals and humans. Since the eye is immune privileged [21], a weak immune reaction is trigged when a virus is injected in the eye [8], reducing the side effects of gene therapy and increasing transfection

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efficiency. In this review, we first discuss current advances in the field of ocular gene therapy and how they necessitate improved diagnostics.

1 Gene therapy

1.1 Ocular gene therapy vectors

Gene therapy, or the correction of a genetic default through the introduction of exogenous nucleic acids, was first performed in mammals early in the 1970s using herpes simplex virus to treat mouse cells with a thymidine kinase enzyme deficiency [22]. The retina of the eye has several advantages for the application of gene therapy. It is immune isolated, and is visible and accessible to clinicians. This allows for relatively easy delivery of vector and non-invasive monitoring after application of the therapy [23]. Further, the compartmentalization of the eye allows for highly localized administration of vectors, typically in the form of modified viruses used to deliver genetic material of interest. These features minimize the risk of serious complications in ocular gene therapy. Indeed, despite numerous clinical trials, serious systemic problems in humans after ocular gene therapy have yet to be observed.

A major breakthrough occurred with the discovery of Adeno-Associated Virus (AAV) as an effective transducing vector [24,25]. The DNA of therapeutic AAVs exists in cell as an episome, precluding concerns about insertional mutagenesis and cancer. Further, AAV is not able to self-propagate, and generates a minimal immune response. Eleven different serotypes of AAV have been discovered so far [26], and modifications to these serotypes have further added to the variety of vectors [27,28]. Targeting studies, typically using GFP, found that various forms of AAV are able to transduce retinal ganglion cells [29,30], the retinal pigment epithelium(RPE) [4,31] and the inner retina [32]. In summary, through both natural variation and directed modification, AAV vectors currently exist with the capability of modifying most retinal cell types, including those most commonly associated with genetic retinal disease. This host of vectors has been utilized for gene therapy both in animals and humans.

1.2 Animal studies

Mammal models of retinal disease have existed since the 1970s [10,11]. The 1990s saw the first successful application of ocular gene therapy *in vivo*, which effectively delayed the onset of photoreceptor death in a mouse model of Retinitis Pigementosa (RP) with mutations in the enzyme βPDE [12]. These rd1 mice were administered sub-retinal injection of AAV2 containing wild type murine βPDE, resulting in increased photoreceptor longevity. This and other early work showed the feasibility of gene therapy for recessive ocular diseases [13], encouraging further work in the field.

Substantial progress has subsequently been made through improvements in viral vectors and delivery systems, leading to an actual improvement of vision, rather than simply delayed degeneration [14,15]. For example, gene replacement was recently performed for the rd10 mouse model using AAV8. All treated eyes showed signs of improved visual function, including on an ERG and spectral domain optical coherence tomography, and benefits remained for at least six months [33]. AAV8 was used to successfully treat MERTK rats, which normally have RP, resulting in improved vision for at least eight months [34]. Gene replacement has succeeded in treating recessive retinal disorders in large animals, from dogs [35] to non-Human primates [36].

As illustrated by these cases, gene replacement in recessive disorders has been highly successful. Additionally, successful ocular gene therapies resulting in improved vision have been performed in animal models of dominant (RP) [37], X-linked (retinoschisis, RP) [38,39], mitochondrial (Leber's hereditary optic neuropathy) [40], and even complex retinal diseases (age-related macular degeneration (AMD)) [41].

1.3 Human gene therapy

Animal studies have led to a renaissance in human gene therapy, with the advent of clinical trials for the treatment of Stargardts, Leber's Congenital Amaurosis (LCA), AMD and RP [2–4]. In 2008, three independent studies completed phase I clinical trials of used AAV2-driven gene replacement to treat retinal degeneration caused by RPE65 mutations [5–7]. The majority of participants reported improved vision. Further, re-administration of the therapeutic vector does not appear to reduce its efficacy, making gene therapy a potential long-term solution for both eyes [8].

Stem cell therapies, which may function on highly degenerated tissues, are also undergoing clinical trials [42]. They operate by replacing damaged cell layers with new, undamaged tissue [43]. This could be done by converting cultured patient cells into Induced Pluripotent Cells (IPCs), which could then be differentiated into retinal precursor cells [44]. Optimally, gene therapy would be performed on the IPCs prior to their transplantation [45] to address the genetic defect in the patient's original tissue. As in traditional gene therapy, application of this step would require high confidence molecular diagnosis, but it would allow restoration of vision in cases with highly degenerate retinas.

In summary, gene therapy for a wide range of ocular disorders is rapidly becoming a clinical reality. While gene therapy is on the road to success, a pre-requisite for its use is discovering the mutations that are causing disease in patients [46]. Therefore, obtaining a complete list of genes underlying the disease and accurately identifying disease causing mutations in each patient are prerequisites for per-

sonalized treatment.

2 Disease gene cloning through next-generation sequencing (NGS)

In the past two decades, more than 185 genes associated with inherited eye diseases have been identified [17] (Figure 1). However, much of this work depended on family based linkage analysis and positional cloning [47,48]. With limited numbers of large disease pedigrees, it is becoming increasingly difficult to pinpoint novel genes using these methods. This challenge can be addressed by sequencing the genomes of patients with a shared disease, with the goal of finding shared rare mutations.

The first case of successful application of this method was in 2009, when mutations in DODH were identified as the cause of Miller syndrome by sequencing just seven individuals, of which four were affected [49]. Since 2009, more than 100 genes responsible for Mendelian disorders have been identified through NGS based methods [50]. In ocular disease, numerous genes have been identified using NGS [51–53]. This process is becoming increasingly robust as larger control databases and more advanced bioinformatics techniques are developed. Details about NGS based gene identification and the resultant findings can be found in an excellent review, which concluded that WES will be an important tool in future gene cloning efforts [50].

Indeed, a recently published theoretical model indicates that sequencing a reasonably sized patient cohort would allow identification of nearly all genes causing a significant fraction of cases of the disease. For example, by sequencing 200 patients with the same recessive Mendelian disorder, >90% power can be achieved to identify genes causing the disease in at least 5% of the affected population (Figure 2) [54]. Therefore, with the progress of large scale projects targeted to Mendelian disorders (http://www.genome.gov/27546192) and rapid accumulation of NGS sequencing results, we expect that the vast majority of Mendelian disease genes can be identified in the near future. This success, however, will cause a rapid increase in the number of genes that must be surveyed to obtain a molecular diagnosis.

3 Molecular diagnosis in retinal disease

Finding causative mutations for retinal disease can be difficult due to the large number of genes responsible, as well as the complexity of the genotype phenotype relationship in these disorders. For example, LCA is caused by eighteen genes [53,55–72], and mutations in these genes can also cause RP, vitreoretinal degeneration, pigmented paravenous chorioretinal atrophy, Senior-Loken syndrome, congenital cone-rod synaptic disease, Joubert syndrome, Meckel syndrome, cone-rod dystrophy, and microphthalmia [73]. New connections are still being made [72,74]. Nonetheless, an accurate molecular diagnosis is essential, not only for gene therapy but also for genetic counseling, pathway specific drug prescription [16], and prognostics. The two most commonly used clinical mutation identification tools are Sanger sequencing and Allele specific Primer EXtention (APEX) arrays.

Sanger sequencing is considered to be the golden standard for mutation identification due to its accuracy. However, because Sanger sequencing is labor intensive and has high per-base sequencing cost, it can be only effectively used to scan a limited number of exons for mutations, or to confirm mutations found by other methods. Given that 185 retinal disease genes have been identified to date, Sanger sequencing every exon potentially contributing to retinal disease is prohibitively costly and time-consuming. This difficulty drove the development of high throughput, array based genotyping methods for retinal disease.

APEX is an array based SNP genotyping technology [75] that is used to sequence the base pair following a designed oligonucleotide of choice. Since each spot on the array only sequences a single base pair, only known pathogenic bases are targeted. The current Asper Ophthalmics LCA array includes 780 disease-associated sequence variants identified in 15 LCA and early-onset RP genes [76]. This method is accurate, but is only able to detect novel mutations if they occur at the same position as a known mutation. Further, only a subset of all known LCA genes and mutations are tested. This process generates a successful diagnosis in ~17%–32% of LCA cases [77,78].

Similarly, three Asper Ophthalmics APEX panels exist for RP, each for a separate form of inheritance. For example, the latest dominant RP panel offered by Asper provides simultaneous detection of 414 known disease-associated variants in 16 genes, while their recessive RP panel screens 594 known mutations in 19 genes [79]. APEX based diagnostics greatly increase diagnosis efficiency when screening a large number of mutations from multiple genes, allowing for high throughput analysis. However, since current arrays can only detect known mutations in a subset of known RP genes, successful diagnosis is only achieved in 15% of all RP cases [80,81]. Further, performing the test requires knowledge about the inheritance pattern of the patient, which is only available for about 50% of patients [82].

Molecular diagnosis of patients using current methods is either prohibitively expensive or suffers from very low accuracy. In the following section, we will review molecular diagnosis using NGS based strategies for three common retinal diseases: LCA, RP, and Stargardt disease.

3.1 NGS based molecular diagnosis in Leber's Congenital Amaurosis

LCA is the most extreme form of inherited retinal disease, as LCA patients typically have severe visual impairment or

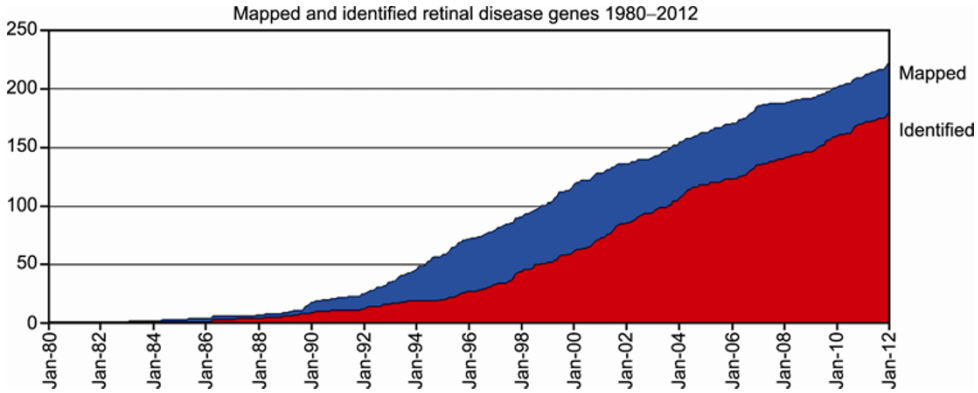

Figure 1 The number of known retinal disease genes (red) and mapped loci (blue) responsible for retinal disease, graphed over time. Modified from: RetNet, http://www.sph.uth.tmc.edu/RetNet/.

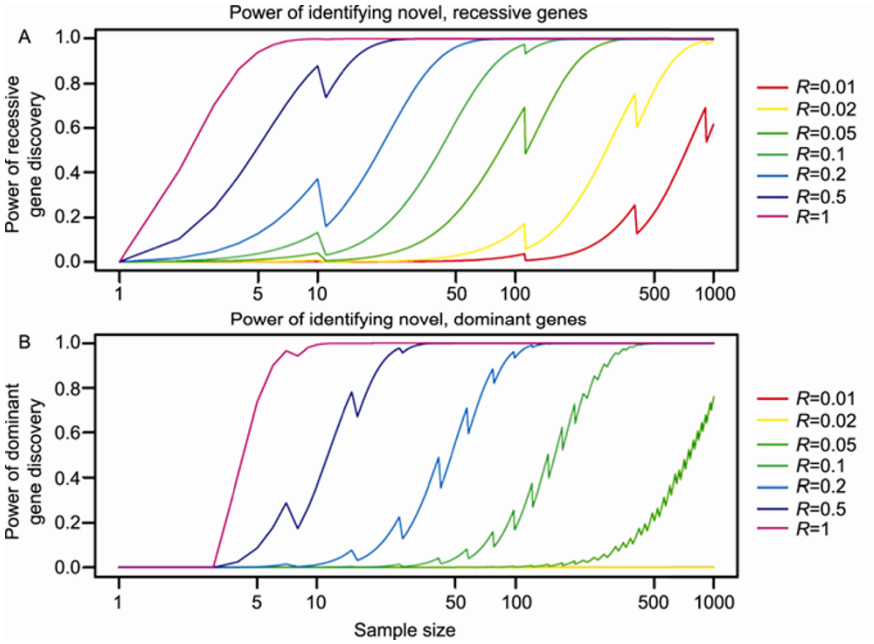

Figure 2 The calculated power with a varying fraction of patients for which a given locus is responsible (R), ranging from 0.01 to 1, is shown. A, Power for detecting a recessive gene. B, Power for detecting a dominant gene. Other parameters are fixed to the default values: number of mutations after filtering=300; total number of genes assayed=20000; sensitivity of detecting mutations=0.8; and the causative mutation occurs in an average sized gene. Note that power does not always increase monotonously with sample sizes (zigzag line patterns). The loss of power upon increase of sample size is related to discrete changes in the significance level cutoff t_α of the test and thus very small test size, since the distribution of the test statistic is discrete. Modified from: Zhi D and Chen R. Statistical guidance for experimental design and data analysis of mutation detection in rare monogenic mendelian diseases by exome sequencing. *PLoS One*, 2012, 7(2): e31358.

blindness within the first year of life. Other symptoms include congenital nystagmus, defective pupillary responses, and a reduced signal in electroretinograms (ERG) [83]. LCA is estimated to affect one in every 30000–80000 individuals and accounts for ~5% of all retinal dystrophies [84,85]. It is estimated that about 70% of European LCA cases are explained by known LCA genes [18], though this number is expected to be lower for different ethnicities [86,87].

Interestingly, mutations in the same gene can cause either syndromic or non syndromic eye disease. For example, mild alleles in CEP290 cause LCA, while complete loss of CEP290 function leads to Joubert syndrome [58]. Also, the recently identified LCA gene KCNJ13 was previously reported to cause vitreoretinal degeneration [72], a related but distinct disorder. These findings underscore the genetic heterogeneity and difficulty of genetic diagnosis in LCA.

Compared with microarray based methods, NGS has a lower per base cost and is able to detect novel mutations and novel genes. When preceded by a capture array, which specifically isolates DNA of interest, NGS can efficiently sequence every base pair in a large area of interest. This flexibility allows NGS to frequently identify novel mutations [88,89] in inherited human diseases. Indeed, Frauke

Coppieters et al. recently diagnosed LCA patients using DNA capture combined with NGS. They were able to confirm previously identified mutations, and found the causal mutations missed by arrays in 3 out 17 patients [90], an increase in diagnosis rate of over 17%.

Due to the genetic heterogeneity of LCA and low rates of diagnosis, it is evident that a portion of LCA is caused by mutations outside of known eye LCA genes. The high throughput nature of capture-NGS can be used to efficiently query large sets of genes to search for this missing inheritance. Indeed, utilizing a capture chip targeting all known retinal disease genes, recent work in our lab shows that a high percentage of LCA cases are indeed caused by mutations in genes known to cause retinal disease, but previously unlinked with LCA (unpublished data).

In summary, NGS based genetic diagnosis of LCA has several advantages over previous methods, but has the added challenge of properly interpreting NGS data. Also, due to LCA's genetic heterogeneity, comprehensive screening every base in all known retinal disease genes is desirable. Currently, this can only be efficiently achieved with NGS.

3.2 NGS based molecular diagnosis in RP

Retinitis pigmentosa (RP) is a progressive retinal degeneration, affecting about 1 in 4000 people worldwide [91]. Initially, rod photoreceptor cells begin to die. Patients at this stage develop early onset night blindness and tunnel vision. As the disease develops, the loss of rod cells begins to damage cone photoreceptors. Their degeneration causes a reduction of central and color vision and may lead to complete blindness. The retinal pigment epithelium (RPE) is the third major type of cell that degenerates over the course of RP, in response to loss of photoreceptor cells. RPE cells release pigment granules as they degenerate, which often accumulate in a bone spicule configuration that further occludes vision.

RP is highly heterogeneous, with autosomal recessive (ar), autosomal dominant (ad), X-linked, digenic, and mitochondrial forms. To date, 52 genes functioning in diverse biological pathways have been linked to RP [92]. Among them, genes involved in the phototransduction cascade account for a major portion of RP cases. Mutations in phototransduction-related genes RHO and PDE are responsible for 25% adRP cases and 8% of arRP cases, respectively [93,94]. Because of its genetic heterogeneity, an accurate molecular diagnosis is challenging. Even with decades of improvement, current diagnostics including Sanger sequencing and APEX still have many limitations.

Recent developments in NGS and DNA capture technology provide a potential new approach to molecular diagnosis in RP. Indeed, it has been shown to have many advantages over current diagnostic methods [19,20]. First of all, NGS based molecular diagnosis of RP is the most comprehensive molecular diagnostic method available. By screening of both known and novel mutations in all known RP genes simultaneously, NGS can achieve a significantly higher rate of successful diagnosis. For example, in two recent studies, about 150 RP patients with a variety of inheritance forms were examined by NGS based molecular diagnosis, revealing an overall diagnosis rate of about 50% [19,20]. Secondly, some RP cases follow a digenic inheritance model [92], and approximately 50% of cases have an unknown inheritance model [82]. NGS can easily be applied to these patients in a single step, making it more applicable than arrays. Finally, the massive sequencing capacity of current NGS machines along with advanced molecular bar-coding technology enables sequencing of multiple samples in parallel [19,20,95–97]. This significantly improves throughput while reducing time and cost, making NGS an ideal diagnostic platform.

3.3 NGS based molecular diagnosis in Stargardt disease

Stargardt disease is a form of early onset macular degeneration affecting at least 1 in 10000 people [98], with approximately 31000 affected in the US alone. In Stargardt disease, there is a relatively fast degeneration of the macula caused by the buildup of oily waste deposits called lipofusin (comprised largely of A2E, a vitamin A derivative) in the retinal pigment epithelium (RPE) cell layer. This limits interactions between photoreceptors and the RPE, hampering the ability of photoreceptors to uptake nutrients and perform the visual cycle, leading to their death.

A Stargardt phenotype is only known to be caused by mutations in three genes; ABCA4 [99], ELOVL4 [100,101], and PROM1 [102]. Of these, ABCA4 is by far the most common cause, and the only known gene for recessive Stargardt disease. ABCA4 functions as a flippase for N-retinylidene-phosphatidylethanolamine and phosphatidylethanolamine, moving these compounds from the luminal to cytoplasmic side of the photoreceptor outer segment discs [103]. Loss of this flippase function leads to the toxic buildup of lipofusin [104]. Mutations in ABCA4 also cause a large fraction of RP and cone-rod dystrophy cases, and as a result ABCA4 is a very well studied gene [98,105].

It might seem that Stargardt disease is a poor choice for NGS based diagnosis, as the power of NGS is not as easily justified in a disease that is only typically caused by mutations in one gene. However, even with a huge number of known exonic variants (>600 on the current diagnostic chip), homozygous or compound heterozygous disease causing mutations in ABCA4 are found in only ~30%–40% of patients using APEX arrays [106,107]. Using NGS based diagnosis for ABCA4, a group was able to identify mutations in 48% (73/142) of their patients [88], while a more recent study using NGS was able to solve 33% of cases that remained unsolved after use of an array [89]. This increase in accuracy is due to the ability of NGS to identify novel, rare,

detrimental alleles. Polymorphic loci in ABCA4 are 9–400 times as common as in other retinal disease genes [108], generating a lot of variety that can only currently be resolved through sequencing. Thus, even in monogenic diseases NGS can offer advantages over array based diagnostic methods.

Regardless of the method used to identify exonic mutations, a molecular diagnosis in Stargardt disease remains elusive in a significant fraction of cases. One explanation for this phenomenon is that intronic, regulatory and/or structural variations, including copy number variations (CNVs) and insertion/deletion mutations, account for a large portion of disease alleles. This hypothesis has been supported by microarray studies, which found ABCA4 haplotypes segregating with disease in families with no identified coding mutations [109]. The genomic size of the ABCA4 locus is over 128 kb, making it far too large for it to be efficiently Sanger sequenced. However, NGS, through the use of a capture array, could be used to probe the entire genomic region around ABCA4. Understanding the effects of extra-exonic mutations is a topic of current research [110]. Improvements are also being made in the ability of NGS to detect large deletions and CNVs [111].

As our knowledge about other retinal diseases grows, it is likely that they will end up like ABCA4 in that many cases of the disease will not be explainable by protein coding variation alone. Improved molecular diagnosis, and its clinical advantages, will necessitate sequencing of large swaths of the human genome, including the regions containing all known retinal disease genes. By moving toward NGS based diagnosis, it is possible to both improve the efficiency of probing exonic regions and open up whole new areas to directed analysis.

4 Perspectives and conclusion

Maximal accuracy is a requirement for clinical diagnostics, as people base major life decisions on the results of diagnostic tests. As shown in the three cases above, NGS improves the accuracy of molecular diagnosis. However, NGS is currently more expensive per patient than arrays (though the price is rapidly falling) and standards for NGS data analysis are still under development. As more causative mutations are found and included on diagnostic arrays, their accuracy should increase. Will arrays eventually become as accurate as NGS?

One way to consider this question is to graph the number of novel mutations found versus the number of people sequenced. If the slope of this line remains high after a large number of people have been sequenced, it would indicate that personal mutations are common, and argue that novel mutations will continue to be found for some time to come. Disease loci may be in this situation, as purifying selection is predicted to keep pathogenic allele frequencies low. This question was addressed earlier this year in a predominantly European cohort of 14002 people. They were analyzed for mutations in 202 drug-targetable genes of clinical significance, and it was found that the rate of observing novel non-synonymous variants was almost constant with increasing sample size. This finding supports the hypothesis that a collection of rare variants is the main driver of disease [112], and that arrays may never be able to include a majority of disease causing variants (Figure 3). As a result, NGS will likely sustain higher accuracy than array based methods in the long term. Aside from accuracy, numerous concerns remain about clinical NGS. These include the cost, infrastructure, business model that will be used, how unclear mutations will be handled, issues regarding the storage and use of genetic information, database management, and the development of standardized bioinformatics tools for sequence data analysis. A recent report by the National Health Service of England highlighted many of these concerns, and proposed the creation of local diagnostic sequencing centers that would service nearby hospitals as a business model [113].

Work is currently underway to develop standardized bioinformatics pipelines to ensure repeatability and reliability in NGS based diagnosis and proper handling of genomic data for large scale use [114]. The cost of NGS has plummeted since its introduction [115], and it is likely that the cost of sequencing will soon be comparable to the cost of other diagnostic methods. Therefore, we expect NGS to become common place in clinical diagnostics in this decade

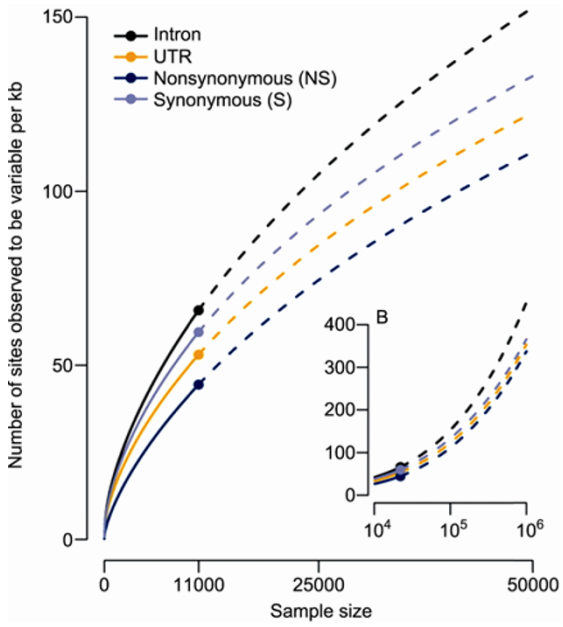

Figure 3 Observed polymorphic loci as sample size increases. A, Observed number mutations as sample size increases. The dots are the observed data, while the dotted lines and solid lines are based on jackknife projections and hypergeometric expectations, respectively. B, A log scale graph allows visualization of up to one million people sequenced. From: Nelson M R, et al., "An abundance of rare functional variants in 202 drug target genes sequenced in 14002 people". *Science*, 2012, 337(6090): 100-104. Reprinted with permission from AAAS.

due to its ability to simultaneously assay broad swaths of the genome with great accuracy in a high throughput manner. NGS is poised to lay the groundwork for personalized medicine by identifying disease causing genes and patient's individual mutations responsible for their phenotypes.

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