Whole-exome sequencing and its impact in hereditary hearing loss

TAHIR ATIK[1,2], GUNEY BADEMCI[1], OSCAR DIAZ-HORTA[1], SUSAN H. BLANTON[1] AND MUSTAFA TEKIN[1]*

[1]*Dr. John T. Macdonald Foundation Department of Human Genetics and John P. Hussman Institute for Human Genomics, Miller School of Medicine, University of Miami, USA*
[2]*Division of Genetics, Department of Pediatrics, Ege University School of Medicine, Izmir, Turkey*

Summary

Next-generation sequencing (NGS) technologies have played a central role in the genetic revolution. These technologies, especially whole-exome sequencing, have become the primary tool of geneticists to identify the causative DNA variants in Mendelian disorders, including hereditary deafness. Current research estimates that 1% of all human genes have a function in hearing. To date, mutations in over 80 genes have been reported to cause nonsyndromic hearing loss (NSHL). Strikingly, more than a quarter of all known genes related to NSHL were discovered in the past 5 years via NGS technologies. In this article, we review recent developments in the usage of NGS for hereditary deafness, with an emphasis on whole-exome sequencing.

1. Introduction

DNA sequencing has been one of the most important techniques in medical research and genetic diagnostics since chain termination sequencing was first described 38 years ago (Sanger *et al.*, 1977). Subsequently, PCR was introduced by Mullis *et al.* (1986), broadening its applications. Automated Sanger sequencers based on capillary electrophoresis were an integral part of The Human Genome Project completed in 2003. The first human genome sequenced was the result of over 10 year's effort at an estimated cost of $2.7 billion (Lander *et al.*, 2001; McPherson *et al.*, 2001; Sachidanandam *et al.*, 2001). Automated Sanger sequencing, also called 'first-generation DNA sequencing', is the most widely used technology worldwide today for variant detection through sequencing. Although currently the gold standard for DNA sequencing, there are some limitations in using this method for high-throughput applications, including read length, runtime and per base cost (Rizzo & Buck, 2012). Fortunately, a high-throughput, powerful technology known as next-generation sequencing (NGS) (or massively parallel sequencing (MPS)) has been developed over the past 10 years. This revolutionary sequencing technique is capable of sequencing millions of small fragments covering the whole genome or large regions of interest, such as the entire coding portion of the genome (i.e. exome), at a reasonable cost and reduced runtime compared to Sanger sequencing (Yan *et al.*, 2013 *a*; Rabbani *et al.*, 2014).

Elucidation of the genetic basis of human diseases is vital for understanding the underlying pathology, making an early diagnosis, developing prevention and/or better treatment regimens, and improving genetic counselling (Gilissen *et al.*, 2012). Although traditional laboratory approaches, including karyotyping and copy number variation (CNV) analysis, and computational methods, including linkage and association, have led to great insights into human diseases over the past few decades, there is still a substantial gap between disease phenotype and the underlying genetic involvements (Bamshad *et al.*, 2011; Gilissen *et al.*, 2011). Limiting elements of traditional gene-discovery strategies, such as the necessity of large families, and the existence of reduced penetrance, variable expressivity and locus heterogeneity have always been a problem for geneticists (Bamshad *et al.*, 2011). The development of NGS, however, has made the identification of causative genes easier, even for small families and diseases with extensive locus heterogeneity (Duman & Tekin,

* Corresponding author: Mustafa Tekin, 1501 NW 10th Avenue, BRB-610 (M-860), Miami, FL 33136, USA. Tel: 305-243-2381. E-mail: mtekin@miami.edu

2012). Furthermore, NGS can also be used to analyse genomic functions through transcriptome, methylome and chromatin structure studies.

Hearing loss (HL) is the most common sensory deficit, affecting millions of people worldwide, with an incidence of 1/1000 newborns in the US. At least half of congenital deafness is due to genetic factors, the vast majority of which is monogenic. Autosomal recessive, autosomal dominant and X-linked inheritance are observed in 77, 22 and 1% of genetic cases, respectively (Morton, 1991). Approximately 70% of congenital deafness is nonsyndromic (nonsyndromic hearing loss; NSHL), while the remaining 30% is comprised of many different syndromes in which deafness is a feature (Shearer & Smith, 2012; Yan *et al.*, 2013 *a*). Current research estimates that 1% of all human genes have a function in hearing (Teek *et al.*, 2013). To date, mutations in over 80 genes, with more than 1000 mutations, have been found to cause NSHL making hereditary hearing loss one of the most genetically heterogeneous traits (http://hereditaryhearingloss.org). Except for those recurrently reported in a few genes, such as *GJB2* (MIM 121011) and *SLC26A4* (MIM 605646), most deafness mutations are extremely rare and are only seen in either a single or a very few families (Diaz-Horta *et al.*, 2012).

Here, we review recent developments on the usage of NGS for hereditary HL, with an emphasis on whole-exome sequencing (WES). The extreme genetic heterogeneity of NSHL makes it the ideal disorder to use to demonstrate the full potential of NGS.

2. NGS platforms and techniques

While there are five commercially available NGS platforms in the marketplace: Roche/454 FLX, the Illumina HiSeq Series, the Applied Biosystems (ABI) SOLiD Analyzer, the Polonator G.007 and the Helicos HeliScope, the first three platforms currently dominate the area (Yan *et al.*, 2013 *a*). Although these platforms each have their own strengths and weaknesses originating from the technologies used, they all include three main stages: template preparation, sequencing/imaging and data analysis. Template preparation is the first step and determines the part of the genome to be sequenced, i.e. whole-genome sequencing (WGS), WES or targeted next-generation sequencing (Yan *et al.*, 2013 *a*). The first platform introduced, Roche/454 GS FLX, uses pyrosequencing. When DNA polymerase incorporates a nucleotide into the growing DNA strand and ATP hydrolysis occurs, the pyrophosphate release is recorded (Shearer *et al.*, 2011). The Illumina platform, based on cyclic reversible termination (CRT) technology, is the most widely used NGS platform. In CRT, fluorescent labelled chain-terminating nucleotides are incorporated into the growing strand, caught and imaged by the sequencer, and then the fluorescent terminator is cleaved off the nucleotide (Ju *et al.*, 2006; Shearer *et al.*, 2011). The SOLiD platform uses sequence-by-ligation (SBL) technology. A fluorescently labelled probe hybridizes to the DNA template to be sequenced. The probe then is joined to the growing strand by DNA ligase, and then imaged by the sequencer (Shearer *et al.*, 2011).

3. Why WES?

Nowadays, sequencing the whole genome is becoming available for wider usage; however, it was not practical until recently because of cost and the amount of data produced. To discover the genes causing Mendelian diseases, especially those having genetic heterogeneity, WES represents a relatively easy-to-use alternative method in the research area (Yan *et al.*, 2013 *a*). While the coding parts of the human genome, i.e. exons of (most) genes or the exome, constitute only about 1% of the entire human genome, 85% of mutations known to cause Mendelian diseases are located in the coding region or in canonical splice sites (Choi *et al.*, 2009). The targeted genomic enrichment required for WES has some challenges, such as incomplete knowledge about all truly protein-coding exons, variable efficiencies of capture probes used and targeting sequence issues (e.g. microRNAs, promoters, pseudogenes, repetitive elements and ultra-conserved elements) (Bamshad *et al.*, 2011). Despite these limitations, exome sequencing has clearly proven to be a powerful tool for discovering the underlying genetic etiology of known or suspected Mendelian disorders. Moreover, by increasing read length and coverage, these problems seem relatively likely to be solved.

4. Data analysis

On average, WES identifies approximately 22 000 single nucleotide variants (SNVs) in a sample. More than 95% of these variants are already known polymorphisms. Strategies for identifying causal SNVs vary depending on a number of factors such as the putative mode of inheritance of the trait, the pedigree structure and the presence of locus heterogeneity in the trait (Bamshad *et al.*, 2011).

Analysis of the WES data begins with filtering out the variants against a set of polymorphisms that are frequent ($> 0.5\%$) in public (e.g. dbSNP and 1000 Genomes Project) or internal databases (e.g. GEM. App) (Gonzalez *et al.*, 2013). This step narrows the number of variants to a manageable fraction (2% on average) of the SNVs identified in an individual. Variations in conserved regions, their functional class (frameshifts, nonsense, splice site) and missense are prioritized in descendant order. Candidate variants
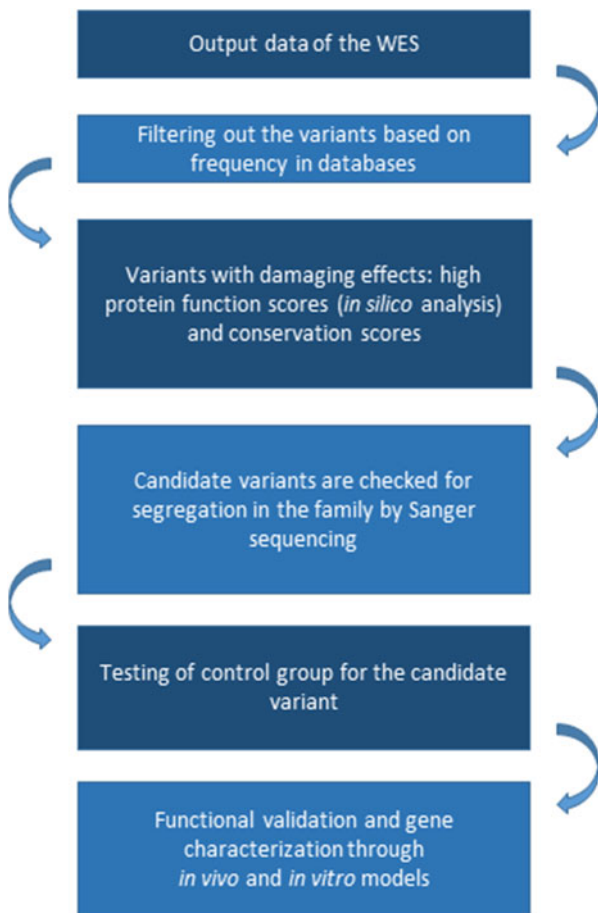
Fig. 1. Typical workflow for gene discovery using whole-exome sequencing. WES, whole-exome sequencing.

are also prioritized according to predicted effects on the protein function (e.g. SIFT (Kumar *et al.*, 2009) and PolyPhen2 (Adzhubei *et al.*, 2010)) and conservation scores (e.g. GERP (Davydov *et al.*, 2010) and PHAST (Hubisz *et al.*, 2011)) (Bamshad *et al.*, 2011).

Analysis of the segregation of candidate variants in the family is also an essential filter, even if mapping data are not available. Sequencing the two most distantly related individuals with the phenotype of interest can substantially reduce the genomic search space for candidate causal alleles. Multiplex families and/or inbred families are more informative than simplex families for inherited causes (Bamshad *et al.*, 2011) (Fig. 1).

5. Gene discovery in NSHL: the impact of WES

Mutations in *POU3F4* (MIM 300039), which was mapped to the X-chromosome in 1988 (Wallis *et al.*, 1988), were identified in 1995 (Wallis *et al.*, 1988; de Kok *et al.*, 1995). It was the first gene discovered as a cause of NSHL. In 1992, the first autosomal dominant locus was mapped to chromosome 5q31 (Leon *et al.*, 1992). Five years later, a mutation was identified in *DIAPH1* at this locus (MIM 602121) (Leon *et al.*,

1992; Lynch *et al.*, 1997). The first locus for autosomal recessive NSHL was mapped to 13q12 (Guilford *et al.*, 1994), which subsequently led to the identification of mutations in *GJB2* (MIM 121011) (Kelsell *et al.*, 1997). This gene is considered to be responsible for up to 50% of autosomal recessive NSHL in childhood in many parts of the world (Guilford *et al.*, 1994; Popov *et al.*, 2014). Prior to 2010, despite intense efforts by leading laboratories over nearly two decades, only 60 deafness genes had been discovered. Since 2010, by employing NGS technologies, 21 genes have been added to the NSHL gene list (http://hereditaryhearingloss.org) (Table 1 and Fig. 2).

In 2010, the first example of NGS used to identify a Mendelian disease gene was the application of exome sequencing to reveal a mutation in *DHODH* (MIM 126064) as the cause of Miller syndrome (MIM 263750) (Ng *et al.*, 2010). The first gene associated with NSHL identified through NGS was *TPRN* (MIM 613354), which causes NSHL at the DFNB79 locus. This locus was mapped to 9q34·3 in a Pakistani family with autosomal recessive NSHL (ARNSHL). In that study, homozygosity mapping was combined with exome sequencing to identify the causative mutation (Rehman *et al.*, 2010). This groundbreaking report was closely followed by another study that used WES as a means to identify a nonsense variant in a novel NSHL gene, *GPSM2* (MIM 609245), in a consanguineous Palestinian family (Walsh *et al.*, 2010). Using WES, mutations in *GPSM2* were later shown to cause Chudley-McCullough syndrome (MIM 604213), characterized by brain anomalies associated with deafness (Doherty *et al.*, 2012). These initial reports were important examples of using WES in ARNSHL. Following these studies, in 2011, Zheng *et al.* (2011) were the first to use NGS to identify a mutation for autosomal dominant NSHL (ADNSHL), *CEACAM16* (MIM 614591).

Analysis of the data obtained through WES is a complicated process, and filters must be applied to simplify the process. Identifying the responsible locus using homozygosity (a.k.a. autozygosity) mapping has been a very efficient way of narrowing the analytic field. With the decreasing cost of WES and understanding of the technology continually increasing, NGS without previously obtained mapping information is positioned to play a pivotal role in novel gene discovery. Behlouli *et al.* (2014) identified a homozygous stop codon mutation in *EPS8* (MIM 600206), encoding an actin-binding protein of cochlear hair cell stereocilia, as the cause of ARNSHL in two affected siblings from a consanguineous family. Similarly, Diaz-Horta *et al.* (2014) reported another novel ARNSHL gene, *FAM65B* (MIM 611410), which encodes a membrane-associated protein of hair cell stereocilia. A homozygous splice site mutation was identified in a large consanguineous

Table 1. *Nonsyndromic deafness genes discovered via whole-exome sequencing*

Hearing loss group	Locus	Gene	Sequencer	Reference
ARNSHL[a]	DFNB79	*TPRN*	Roche 454	(Rehman *et al.*, 2010)
	DFNB82	*GPSM2*[b]	Illumina GAIIx	(Walsh *et al.*, 2010)
	DFNB98	*TSPEAR*	Illumina HiSeq	(Delmaghani *et al.*, 2012)
	DFNB93	*CABP2*	Illumina GAIIx	(Schrauwen *et al.*, 2012)
	DFNB84	*OTOGL*	Illumina HiSeq	(Yariz *et al.*, 2012)
	DFNB89	*KARS*	Illumina HiSeq	(Santos-Cortez *et al.*, 2013)
	DFNB88	*ELMOD3*	Illumina HiSeq	(Jaworek *et al.*, 2013)
	DFNB76	*SYNE4*	Illumina HiSeq	(Horn *et al.*, 2013)
	DFNB49	*BDP1*	SOLiD4	(Girotto *et al.*, 2013)
	N/A[c]	*EPS8*	Illumina HiSeq	(Behlouli *et al.*, 2014)
	DFNB101	*GRXCR2*	Illumina HiSeq	(Imtiaz *et al.*, 2014)
	DFNB86[d]	*TBC1D24*	Illumina HiSeq	(Rehman *et al.*, 2014)
	N/A[c]	*FAM65B*	Illumina HiSeq	(Diaz-Horta *et al.*, 2014)
	DFNB44	*ADCY1*	Illumina HiSeq	(Santos-Cortez *et al.*, 2014)
	DFNB99	*TMEM132E*	Illumina HiSeq	(Li *et al.*, 2014)
ADNSHL[a]	DFNA4	*CEACAM16*	SOLiD4	(Zheng *et al.*, 2011)
	DFNA56	*TNC*	Illumina HiSeq	(Zhao *et al.*, 2013)
	DFNA41	*P2RX2*	Illumina HiSeq	(Yan *et al.*, 2013 *b*)
	DFNA65[d]	*TBC1D24*	Illumina HiSeq	(Azaiez *et al.*, 2014; Zhang *et al.*, 2014)
	N/A[c]	*OSBPL2*	Illumina HiSeq	(Xing *et al.*, 2014)
XNSHL[a]	DFNX4	*SMPX*	Illumina GAIIx	(Schraders *et al.*, 2011; Huebner *et al.*, 2011)
	N/A[c]	*COL4A6*	Illumina HiSeq	(Rost *et al.*, 2014)

[a] ADNSHL, autosomal dominant nonsyndromic hearing loss; ARNSHL, autosomal recessive nonsyndromic hearing loss; XNSHL, X-linked nonsyndromic hearing loss.
[b] *GPSM2* mutations have been subsequently shown to cause Chudley-McCullough syndrome (Doherty *et al.*, 2012).
[c] N/A, not available.
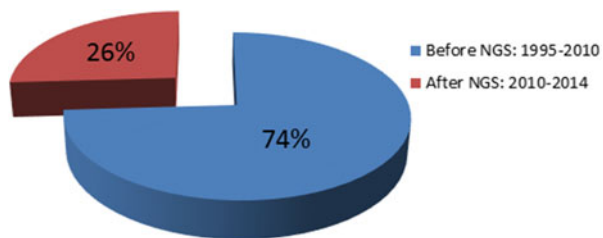[d] Mutations in the *TBC1D24* gene are responsible for ARNSHL or ADNSHL.

Fig. 2. The impact of next-generation sequencing on gene discovery for nonsyndromic deafness. NGS, next-generation sequencing.

family. In both these latter cases, WES was employed without prior studies identifying the loci.

6. Diagnostic usage of NGS in NSHL

In clinical diagnostics for HL, targeted genomic enrichment followed by NGS has been shown to be an efficient strategy. In this scenario, a group of genes or a specific region are sequenced. Brownstein *et al.* (2011), using this strategy, screened 246 genes known to be responsible for human or mouse deafness in 11 probands of Middle Eastern origin and identified causative mutations in six of them. Shearer *et al.* (2013) suggested that this strategy can provide comprehensive genetic testing on a large number of patients with presumed genetic deafness. In their study, they used a panel that included 89 known deafness genes and yielded a diagnostic rate of 31% in ADNSHL and 56% in ARNSHL (Shearer *et al.*, 2013). In another study to investigate the diagnostic utility of targeted genomic enrichment followed by NGS in China, a panel designed to target 80 common deafness genes was used in 12 multiplex families with NSHL and causative variants were identified in four families (Wu *et al.*, 2013). Finally, Vozzi *et al.* (2014) found the causative gene variants in four out of 12 families from Italy and Qatar and confirmed the usefulness of a targeted sequencing approach using a panel including 96 known genes related to HL.

On the other hand, WES has been shown to be effective in identifying causative mutations. In our recent WES study of 20 families prescreened for mutations in *GJB2*, 60% (12/20) of the cases had a mutation in a known gene; we were able to identify the causative mutation in the remaining eight families (Diaz-Horta *et al.*, 2012). While targeted NGS panels provide higher coverage for individual genes, a significant lowering of costs, ease-of-analysis and ease-of-counselling, WES eliminates the need of continued development and validation of custom panels. Importantly, it offers a direct access to novel gene discovery in people who do not have mutations in known deafness genes (Diaz-Horta *et al.*, 2012).

7. Conclusion

NGS is now an accepted clinical and research tool for the study of genetic deafness. It is a powerful approach for identifying patient-specific etiologies in genetically heterogeneous disorders and/or undiagnosed Mendelian phenotypes and has the potential to dramatically change the delivery of patient care. Hereditary HL presents as one of the most suitable disorders for the application of this 'cutting-edge' technology. NGS should be strongly considered, especially in cases where etiological diagnosis is inconclusive following established single gene testing, or when NGS presents as a faster and less expensive option for accurate genetic diagnosis.

Declaration of Interest

None.

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