BMJ Open

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Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2014-007506
Article Type:	Research
Date Submitted by the Author:	22-Dec-2014
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Primary Subject Heading:	Genetics and genomics
Secondary Subject Heading:	Diagnostics, Ear, nose and throat/otolaryngology
Keywords:	GENETICS, OTOLARYNGOLOGY, Adult otolaryngology < OTOLARYNGOLOGY, Paediatric otolaryngology < OTOLARYNGOLOGY, Neurotology < OTOLARYNGOLOGY

DNA sequence analysis and genotype-phenotype assessment in 71 patients with syndromic hearing loss or auditory neuropathy

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Keywords: syndromic, hearing loss, enlarged vestibular aqueduct, Usher syndrome, auditory neuropathy

Word count: 1392

ABSTRACT

Objectives: Etiologic assessment of 71 probands whose clinical presentation suggested a genetic syndrome or auditory neuropathy.

Methods: Sanger sequencing was performed on DNA isolated from peripheral blood or lymphoblastoid cell lines. Genes were selected for sequencing based on each patient's clinical presentation and suspected diagnosis. Observed DNA sequence variations were assessed for pathogenicity by review of the scientific literature and mutation and polymorphism databases, through the use of *in silico* tools including SIFT and PolyPhen, and according to the recommendations of the American College of Medical Genetics and Genomics for the interpretation of DNA sequence variations. Novel DNA sequence variations were sought in controls.

Results: DNA sequencing of the coding and near-coding regions of genes relevant to each patient's clinical presentation revealed 37 sequence variations of known or uncertain pathogenicity in 9 genes from 25 patients. Fourteen novel sequence variations were discovered. Assessment of phenotypes revealed notable findings in 9 patients.

Conclusions: DNA sequencing in patients whose clinical presentation suggested a genetic syndrome or auditory neuropathy provided opportunities for etiologic assessment and more precise genetic counseling of patients and families. The failure to identify a genetic etiology in many patients in this study highlights the extreme heterogeneity of genetic hearing loss, the incompleteness of current knowledge of etiologies of hearing loss, and the limitations of conventional DNA sequencing strategies that evaluate only coding and near-coding segments of genes.

ARTICLE SUMMARY

Strengths and limitations of this study

- As a research study, it was possible to perform DNA sequencing of a greater number of genes for each patient than would have been economically feasible by clinical genetic testing.
- Patients were followed over time allowing ongoing assessment of phenotypes and hearing status, and continuous refinement of suspected etiologies.
- Patients were evaluated using a multidisciplinary team approach which included otolaryngologists, clinical geneticists, audiologists, speech and language therapists and others as appropriate for each patient, thus enhancing phenotypic assessment.
- The small number of patients evaluated in this study limits the number of genetic variants identified.
- This study was not designed to order clinical diagnostic assessments solely for research purposes; as such, assessment of phenotypes and estimations of potential etiologies for hearing loss are limited to what was observed by physicians in the course of routine clinical care for patients with hearing loss.

BACKGROUND

Genetic hearing loss demonstrates extreme locus and allelic heterogeneity[1-4]. More than 400 genetic syndromes include hearing loss as a feature and more than 100 genes and genetic loci have been associated with nonsyndromic genetic hearing loss. Diagnosis of many syndromic forms of hearing loss can be made based on physical findings while diagnosis of many others, espically syndromes with variable, nonspecific or age-related features, is facilitated by genetic testing. Additionally, many causes of nonsyndromic genetic hearing loss demonstrate similar audiometric profiles. Etiologic assessment of nonsyndromic genetic hearing loss is greatly aided by genetic testing[1-4].

In this study, DNA sequencing was performed for 71 probands with hearing loss whose clinical presentation suggested a genetic syndrome or auditory neuropathy. Sequencing of the coding and near-coding regions of genes relevant to each patient's clinical presentation revealed 37 sequence variations of known or uncertain pathogenicity in 25 patients. Fourteen novel sequence variations were discovered. Assessment of phenotypes revealed notable findings in 9 patients.

METHODS

Ethics approval. This research was approved by the Institutional Review Board (IRB) of Baylor College of Medicine. A Federal Certificate of Confidentiality was obtained.

Patients. Patients with hearing loss of suspected genetic etiology were identified through the clinical care centers of Baylor College of Medicine and Texas Children's Hospital. Written informed consent was obtained from all patients or, in the case of minor children, at least one parent or legal guardian. Clinical evaluations of patients were conducted by physicians in accordance with routine clinical care for patients with hearing loss and the physicians' best clinical judgment[5].

Controls. Controls were obtained from the Baylor Polymorphism Resource of Baylor College of Medicine. The control group consisted of ≥50 individuals from each of 4 ancestral groups: African American, Asian, Caucasian, and Hispanic.

Specimen collection and DNA isolation. Blood was collected by peripheral venipuncture for the puroses of DNA isolation and the establishment of lymphoblastoid cell lines. Lymphoblastoid cell lines were established by standard Epstein Barr virus mediated transformation. DNA was isolated from blood samples and cell lines using PUREGENE® DNA Purification Kits (Qiagen, Valencia, CA, USA) for whole blood or cultured cells according to the manufacturer's specifications.

DNA sequencing. Clinical or research-based DNA sequencing of *GJB2* was performed for all patients in this study group. Additional genes were selected for sequence analysis based on clinical findings. Polymerase chain reaction (PCR) amplification and Sanger sequencing of the coding and near-coding

regions of selected genes were performed according to standard protocols. Primer sequences and PCR and sequencing conditions will be provided upon request.

Nomenclature. DNA and protein sequence variations are named according to standard nomenclature recommendations[6].

Interpretation of DNA sequence variations. Observed DNA sequence variations were assessed for pathogenicity by review of the scientific literature and mutation and polymorphism databases,[7-10] through the use of *in silico* tools including SIFT and PolyPhen,[11, 12] and according to the recommendations of the American College of Medical Genetics and Genomics for the interpretation of DNA sequence variations[13]. Novel DNA sequence variations identified in patients were sought in controls.

RESULTS

Sixty seven probands with hearing loss were diagnosed with or suspected of having a genetic syndrome based on clinical observations: 47 patients had enlarged vestibular aqueduct, Mondini malformation or other inner ear malformations; 7 patients had Usher syndrome – 2 with type 1, 5 with type 2; 1 patient had enlarged vestibular aqueducts and Usher syndrome type 2; 6 patients had Waardenburg syndrome – 3 with type 1, 1 with type 4, 2 with peripheral demyelinating neuropathy, central dysmyelination, Waardenburg syndrome, Hirschsprung disease (PCWH); 5 patients had prolonged QT interval – 1 with signs of VACTERL association (vertebral defects, anal atresia, cardiac defects, tracheo-esophageal fistula, renal anomalies, limb defects); and, 1 patient had Cornelia de Lange syndrome. Four probands with auditory neuropathy were also included in this study group.

All patients had clinical or research-based sequencing of *GJB2* which excluded *GJB2* as the cause of their hearing loss. Additional genes for DNA sequence analysis were selected for each patient based on clinical findings. Among the 71 patients in this study group, 37 different DNA sequence variations of known or uncertain pathogenicity were observed in the coding and near coding regions of relevant genes in 25 patients including 1 regulatory, 1 translation start site, 18 missense, 3 nonsense, 1 synonymous, 7 splice site, and, 6 frameshift mutations. Fourteen of the observed variants were understood to be novel at the time this manuscript was written including 5 missense, 2 nonsense, 1 synonymous, 4 splice site and 2 frameshift mutations. Of these 14 novel variants, 7 were interpreted as mutations (1 missense, 2 nonsense, 2 splice site, 2 frameshift), and 7 were deemed to be of uncertain pathogenicity (4 missense, 1 synonymous, 2 splice site) (Table 1). Only 1 of the novel variants discovered was observed in >400 control chromosomes which included at least 100 chromosomes each of African American, Asian, Caucasian and Hispanic ancestry: the *USH2A* p.Thr3976Thr (c.11928G>A) variant was observed in 1 of 106 control chromosomes of Caucasian ancestry.

Table 1. Patient Genotypes.

HUGO gene name *Molecular findings*	Number patients with genotype [*]	Nucleotide variants	Amino acid variants	Interpre -tation	Phase known?	Additional findings/ clinical diagnosis
SLC26A4		RefSeq: NM_000441.1	RefSeq: NP_000432.1			
2 mutations (n=4)	1	c.1-103T>C c. 1246A>C	- p.Thr416Pro	M M	No	Bilateral MON; asymmetric HL†
	1	c.165-1G>A (IVS2-1G>A) c. 1246A>C	- p.Thr416Pro	M M	No	Bilateral MON
	1	**c.765+3A>C (IVS6+3A>C)** c.1001+1G>A (IVS8+1G>A)	- -	**M** M	*In trans*	Bilateral: EVA, SCA†
	1	c.2T>C c.1341+1G>C (IVS11+1G>C)	p.0? -	M M	*In trans*	Bilateral: MON, SCA†
1 Mutation (n=5)	1	c.1-103T>C	-	M		UNI: C-VCA, ANH, HL†
	1	c.707T>C	p.Leu236Pro	M		UNI: EVA, HL†
	1	c.2T>C c.2219G>T	p.0? p.Gly740Val	M VUS	No	Bilateral EVA
	1	c.578C>T **c.691G>C**	p.Thr193Ile **p.Val231Leu**	M **VUS**	No	Bilateral MON; asymmetric HL†
	1	c.1-103T>C c.1790T>C	- p.Leu597Ser	M VUS	*In trans*	Bilateral EVA
≥ 1VUS (n=2)	1	c.17G>T c.1790T>C	p.Gly6Val p.Leu597Ser	VUS VUS	No	Bilateral IEM; UNI HL†
	1	c.706C>G	p.Leu236Val	VUS		Bilateral: MON, SCA†; MCA
OTOF		NM_194248.1	NP_919224.1			
2 mutations (n=1)	1	**c.897+1G>T (IVS9+1G>T)** c.2485C>T	- p.Gln829X	**M** M	No	Auditory neuropathy
1 Mutation (n=1)	1[14]	**c.1172delA** c.1614C>A c.1910T>C c.2401_2402GA>TT c.4216G>A	**p.Lys391ArgfsX31** p.Asn538Lys p.Ile637Thr p.Glu801Leu p.Asp1406Asn	**M** VUS VUS P VUS	No	Auditory neuropathy
USH1C		NM_153676.2	NP_710142.1			
2 mutations (n=1)	1	c.238dupC c.238dupC	p.Arg80ProfsX69 p.Arg80ProfsX69	M M	Presumed homozygote	USH1
CDH23		NM_022124.3	NP_071407.3			
>2 VUS (n=1)	1	**c.3929C>A** **c.4104+4A>T (IVS32+4A>T)** **c.9510+19_9510+25delGGCATCA (IVS67+19_25delGGCATCA)**	**p.Ala1310Asp** - -	**VUS** **VUS** **VUS**	No	USH1, SCA†
USH2A		NM_206933.1	NP_996816.1			
1 Mutation (n=5)	1	c.2299delG **c.1724G>T** **c.11928G>A**	p.Glu767SerfsX21 **p.Cys575Phe** **p.Thr3976Thr**	M **VUS** **VUS**	*In trans* as grouped	USH2
	1	**c.7475C>A** c.9203T>C	**p.Ser2492X** p.Val3068Ala	**M** VUS	*In cis*	USH2
	1	c.920_923dupGCCA	p.His308GlnfsX16	M		USH2
	1	c.2299delG c.3407G>A	p.Glu767SerfsX21 p.Ser1136Asn	M VUS	*In trans*	USH2
	1	c.2299delG	p.Glu767SerfsX21	M		USH2
KCNQ1		NM_000218.2	NP_000209.2			
1 Mutation (n=1)	1[14]	c.572_576delTGCGC	p.Leu191LeufsX91	M		Borderline LQT
SOX10		NM_006941.3	NP_008872.1			
Presumptive mutation (n=2)	1[15]	**c.271_275delCCCGT**	**p.Pro91AlafsX41**	**M**		WS4
	1	**c.1127C>G**	**p.Ser376X**	**M**		PCWH
PAX3		NM_181457.1	NP_852122.1			
1 VUS (n=1)	1	**c.241G>T**	**p.Gly81Cys**	**VUS**		WS1
NIPBL		NM_133433.2	NP_597677.2			
Presumptive mutation (n=1)	1	**c.5378T>G**	**p.Met1793Arg**	**M**	*De novo*	CdLS

Variants understood to be novel at the time this manuscript was written are shown in bold typeface. Abbreviations used: M = pathogenic mutation; P = benign polymorphism; VUS = variant of uncertain pathogenicity; MON = Mondini malformation; HL = hearing loss; EVA = enlarged vestibular aqueduct; SCA = semicircular canal abnormalities; UNI = unilateral; C-VCA = cystic vestibulocochlear anomaly; ANH = auditory nerve hypoplasia; IEM = inner ear malformations; MCA = multiple congenital anomalies; USH(#) = Usher syndrome(type); LQT = prolonged QT interval; WS(#) = Waardenburg syndrome(type); PCWH = peripheral demyelinating neuropathy, central dysmyelination, Waardenburg syndrome, Hirschsprung disease; CdLS = Cornelia de Lange syndrome. *Citations [#] for patients included in cohorts exploring independent research questions. †Atypical phenotype.

As noted with a dagger symbol in the far right column of Table 1, atypical phenotypes were observed in 9 patients. Notably, among the 4 patients carrying 2 mutations in *SLC26A4*, 1 patient was found to have asymmetric hearing loss despite having bilateral Mondini malformation, and 2 patients were found to have bilateral malformations of the semicircular canals. Among the 5 patients carrying 1 mutation in *SLC26A4*, 1 patient had unilateral hearing loss with unilateral cystic vestibulocochlear anomaly and auditory nerve hypoplasia on the same side as the hearing loss, 1 patient had unilateral hearing loss and a unilateral enlarged vestibular aqueduct on the same side as the hearing loss, and 1 patient also carried a novel DNA sequence variant of uncertain pathogenicity and had asymmetric hearing loss despite having bilateral Mondini malformation. Among the 2 patients carrying 1 or more variants of uncertain pathogenicity in *SLC26A4*, 1 patient carrying 2 variants of uncertain pathogenicity had unilateral hearing loss despite having bilateral inner ear malformations involving the vestibule and semicircular canals, and 1 patient carrying a single variant of uncertain pathogenicity had bilateral Mondini malformation with bilateral semicircular canal abnormalities and multiple congenital anomalies of unknown but presumably independent etiology. Additionally, 1 patient with a clinical diagnosis of Usher syndrome type 1 carrying 3 variants of uncertain pathogenicity in *CDH23* had bilateral malformation of the semicircular canals.

DISCUSSION

Briefly, 37 different DNA sequence variations of known or uncertain pathogenicity were identified in the coding and near-coding regions of 9 genes in 25 of 71 patients with hearing loss whose clinical presentation suggested a genetic syndrome or auditory neuropathy. Of these DNA sequence variations, 14 were understood to be novel at the time this manuscript was written. Atypical phenotypes were observed in 9 patients.

This study illustrates the clinical utility of DNA sequencing in patients whose presentation suggests a genetic syndrome or auditory neuropathy. The failure to identify a genetic etiology in many patients in this study highlights the extreme heterogeneity of genetic hearing loss, the incompleteness of current knowledge of etiologies of hearing loss, and the limitations of conventional DNA sequencing strategies that evaluate only coding and near-coding segments of genes.

ACKNOWLEDGEMENTS

The authors acknowledge the efforts of Dr. Reid Sutton, Dr. Carlos Bacino, Dr. Fernando Scaglia, Dr. Ross Tonini, Claudia Emery and Janet Maxian.

COMPETING INTERESTS

The authors have no competing interests to declare.

FUNDING

This work was supported by the Alkek Foundation for Research, Sid Richardson Foundation, Brown Foundation Research and Bintliff Foundation for Research.

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BMJ Open

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Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2014-007506.R1
Article Type:	Research
Date Submitted by the Author:	23-Mar-2015
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Distinct physical findings associated with many syndromic forms of hearing loss direct targeted DNA sequence analysis toward particular genes. For example, enlarged vestibular aqueducts suggest Pendred syndrome and mutations in *SLC26A4*. Retinitis pigmentosa suggests Usher syndrome and mutations in *MYO7A, USH1C, CDH23,USH2A* or other Usher syndrome associated genes. Pigmentary anomalies suggest Waardenburg syndrome and mutations *PAX3, MITF, SOX10* or other Waardenburg syndrome associated genes. Prolonged QT interval suggests Jervell and Lange-Nielsen syndrome and mutations in *KCNQ1* or *KCNE1*. Clinical features of Cornelia de Lange syndrome suggest mutations in *NIPBL, SMC1A* or *SMC3*[1-4]. Numerous other syndrome-gene associations have also been described[1-4].

Auditory neuropathy is a distinct form of hearing loss where the outer hair cells function appropriately but sound is not transmitted properly to brain. Although auditory neuropathy may occur as part of a syndrome, it may also occur as an isolated finding associated with mutations in *OTOF, PJVK* or *DIAPH3*[1-5].

In this study, DNA sequencing was performed for 71 probands with hearing loss whose clinical presentation suggested a genetic syndrome or auditory neuropathy. Sequencing of the coding and near-coding regions of genes relevant to each patient's clinical presentation revealed 37 sequence variations of known or uncertain pathogenicity in 25 patients. Fourteen novel sequence variations were discovered. Assessment of phenotypes revealed notable findings in 9 patients.

METHODS

Ethics approval. This research was approved by the Institutional Review Board (IRB) of Baylor College of Medicine. A Federal Certificate of Confidentiality was obtained.

Patients. Patients with hearing loss of suspected genetic etiology were identified through the clinical care centers of Baylor College of Medicine and Texas Children's Hospital. Parents of patients were offered enrollment in this study where appropriate to clarify their children's genetic test results. Written informed consent was obtained from all study participants or, in the case of minor children, at least one parent or legal guardian. Clinical evaluations of patients were conducted by physicians in accordance with routine clinical care for patients with hearing loss and the physicians' best clinical judgment[6].

Controls. Controls were obtained from the Baylor Polymorphism Resource of Baylor College of Medicine. The control group consisted of ≥50 individuals from each of 4 ancestral groups: African American, Asian, Caucasian and Hispanic.

Specimen collection and DNA isolation. Blood was collected by peripheral venipuncture for the purposes of DNA isolation and the establishment of lymphoblastoid cell lines. Lymphoblastoid cell lines were established by standard Epstein Barr virus mediated transformation. DNA was isolated from blood samples and cell lines using PUREGENE® DNA Purification Kits (Qiagen, Valencia, CA, USA) for whole blood or cultured cells according to the manufacturer's specifications.

DNA sequencing. Clinical or research-based DNA sequencing of *GJB2* was performed for all patients in this study group. Additional genes were selected for sequence analysis based on clinical findings. Polymerase chain reaction (PCR) amplification and Sanger sequencing of the coding and near-coding

regions of selected genes were performed according to standard protocols. Primer sequences and PCR and sequencing conditions will be provided upon request.

Nomenclature. DNA and protein sequence variations are named according to standard nomenclature recommendations[7].

Interpretation of DNA sequence variations. Observed DNA sequence variations were assessed for pathogenicity by review of the scientific literature and mutation and polymorphism databases,[8-11] through the use of *in silico* tools including SIFT and PolyPhen,[12, 13] and according to the recommendations of the American College of Medical Genetics and Genomics for the interpretation of DNA sequence variations[14]. The pathogenicity of previously reported DNA sequence variations was interpreted on the preponderance of evidence from prior reports and the predicted effect on the encoded protein product. Novel DNA sequence variations were interpreted as pathogenic mutations if they predicted nonsense codons or frameshifts followed by nonsense codons, occurred within splice site consensus sequences, were *de novo* changes in autosomal dominant conditions, or occurred at a position where a different nucleotide substitution had previously been reported as pathogenic. Novel DNA sequence variations were interpreted as variants of uncertain pathogenicity (VUS) if they predicted missense or synonmous codons or occured near but not within canonical splice site consensus sequences. Novel DNA sequence variations identified in patients were sought in controls.

RESULTS

Sixty seven probands with hearing loss were diagnosed with or suspected of having a genetic syndrome based on clinical observations: 47 patients had enlarged vestibular aqueduct, Mondini malformation or other inner ear malformations; 7 patients had Usher syndrome – 2 with type 1, 5 with type 2; 1 patient had enlarged vestibular aqueducts and Usher syndrome type 2; 6 patients had Waardenburg syndrome – 3 with type 1, 1 with type 4, 2 with peripheral demyelinating neuropathy, central dysmyelination, Waardenburg syndrome, Hirschsprung disease (PCWH); 5 patients had prolonged QT interval – 1 with signs of VACTERL association (vertebral defects, anal atresia, cardiac defects, tracheo-esophageal fistula, renal anomalies, limb defects); and, 1 patient had Cornelia de Lange syndrome. Four probands with auditory neuropathy were also included in this study group: 3 had no known additional relevant phenotypic findings; 1 had brachycephaly, asymmetric facies and cupped ears.

All patients had clinical or research-based sequencing of *GJB2* which excluded *GJB2* as the cause of their hearing loss. Additional genes for DNA sequence analysis were selected for each patient based on clinical findings. Among the 71 patients in this study group, 37 different DNA sequence variations of known or uncertain pathogenicity were observed in the coding and near-coding regions of relevant genes in 25 patients including 1 regulatory, 1 translation start site, 18 missense, 3 nonsense, 1 synonymous, 7 splice site and 6 frameshift mutations. Fourteen of the observed variants were understood to be novel at the time this manuscript was written including 5 missense, 2 nonsense, 1 synonymous, 4 splice site and 2 frameshift mutations. Of these 14 novel variants, 7 were interpreted as mutations (1 *de novo* missense, 2 nonsense, 2 splice site, 2 frameshift) and 7 were deemed to be of uncertain pathogenicity (4 missense, 1 synonymous, 2 splice site) (Table 1). Only 1 of the novel variants discovered in this study was observed in >400 control chromosomes which included at least 100 chromosomes each of African American, Asian, Caucasian and Hispanic ancestry: the *USH2A*

p.Thr3976Thr (c.11928G>A) variant was observed in 1 of 106 control chromosomes of Caucasian ancestry.

Table 1. Patient Genotypes.

HUGO gene name *Molecular findings*	Number patients with genotype [*]	Nucleotide variants	Amino acid variants	Interpre -tation	Phase known?	Additional findings/ clinical diagnosis	Degree of hearing loss
SLC26A4		RefSeq: NM_000441.1	RefSeq: NP_000432.1				
2 mutations (n=4)	1	c.1-103T>C c. 1246A>C	- p.Thr416Pro	M M	No	Bilateral MON; asymmetric HL†	R: Mi/Mo L: Mo sloping to S
	1	c.165-1G>A (IVS2-1G>A) c. 1246A>C	- p.Thr416Pro	M M	No	Bilateral MON	R: S/P L: S/P
	1	**c.765+3A>C (IVS6+3A>C)** c.1001+1G>A (IVS8+1G>A)	- -	**M** M	*In trans*	Bilateral: EVA, SCA†	R: S/P L: S/P
	1	c.2T>C c.1341+1G>C (IVS11+1G>C)	p.0? -	M M	*In trans*	Bilateral: MON, SCA†	R: P L: P
1 Mutation (n=5)	1	c.1-103T>C	-	M		UNI: C-VCA, ANH, HL†	R: P L: NL
	1	c.707T>C	p.Leu236Pro	M		UNI: EVA, HL†	R: NL L: S/P
	1	c.2T>C c.2219G>T	p.0? p.Gly740Val	M VUS	No	Bilateral EVA	NA
	1	c.578C>T **c.691G>C**	p.Thr193Ile **p.Val231Leu**	M **VUS**	No	Bilateral MON; asymmetric HL†	R: S L: Mo
	1	c.1-103T>C c.1790T>C	- p.Leu597Ser	M VUS	*In trans*	Bilateral EVA	R: P L: P
≥ 1VUS (n=2)	1	c.17G>T c.1790T>C	p.Gly6Val p.Leu597Ser	VUS VUS	No	Bilateral IEM; UNI HL†	R: S/P L: NL
	1	c.706C>G	p.Leu236Val	VUS		Bilateral: MON, SCA†; MCA	R: P L: P
OTOF		NM_194248.1	NP_919224.1				
2 mutations (n=1)	1	**c.897+1G>T (IVS9+1G>T)** c.2485C>T	- p.Gln829X	**M** M	No	Auditory neuropathy	R: S/P L: S/P
1 Mutation (n=1)	1[15]	**c.1172delA** c.1614C>A c.1910T>C c.2401_2402GA>TT c.4216G>A	**p.Lys391ArgfsX31** p.Asn538Lys p.Ile637Thr p.Glu801Leu p.Asp1406Asn	**M** VUS VUS P VUS	No	Auditory neuropathy	R: P L: P
USH1C		NM_153676.2	NP_710142.1				
2 mutations (n=1)	1	c.238dupC c.238dupC	p.Arg80ProfsX69 p.Arg80ProfsX69	M M	Presumed homozygote	USH1	R: P L: P
CDH23		NM_022124.3	NP_071407.3				
3 VUS (n=1)	1	**c.3929C>A** **c.3929C>A** **c.4104+4A>T (IVS32+4A>T)** **c.4104+4A>T (IVS32+4A>T)** **c.9510+19_9510+25delGGCATCA (IVS67+19_25delGGCATCA)** **c.9510+19_9510+25delGGCATCA (IVS67+19_25delGGCATCA)**	**p.Ala1310Asp** **p.Ala1310Asp** - - - -	**VUS** **VUS** **VUS** **VUS** **VUS** **VUS**	Presumed homozygote	USH1, SCA†	R: P L: P
USH2A		NM_206933.1	NP_996816.1				
1 Mutation (n=5)	1	c.2299delG **c.1724G>T** **c.11928G>A**	p.Glu767SerfsX21 **p.Cys575Phe** **p.Thr3976Thr**	M **VUS** **VUS**	*In trans* as grouped	USH2	R: Mo sloping to S L: Mo sloping to S
	1	**c.7475C>A** c.9203T>C	**p.Ser2492X** p.Val3068Ala	**M** VUS	*In cis*	USH2	R: Mo sloping to S L: Mo sloping to S
	1	c.920_923dupGCCA	p.His308GlnfsX16	M		USH2	R: S/P L: S/P
	1	c.2299delG c.3407G>A	p.Glu767SerfsX21 p.Ser1136Asn	M VUS	*In trans*	USH2	NA
	1	c.2299delG	p.Glu767SerfsX21	M		USH2	NA
KCNQ1		NM_000218.2	NP_000209.2				
1 Mutation (n=1)	1[15]	c.572_576delTGCGC	p.Leu191LeufsX91	M		Borderline LQT	R: S/P L: S/P
SOX10		NM_006941.3	NP_008872.1				
Presumptive mutation (n=2)	1[16]	**c.271_275delCCCGT**	**p.Pro91AlafsX41**	**M**		WS4	R: S/P L: S/P
	1	**c.1127C>G**	**p.Ser376X**	**M**		PCWH	R: P L: P
PAX3		NM_181457.1	NP_852122.1				
1 VUS (n=1)	1	**c.241G>T**	**p.Gly81Cys**	**VUS**		WS1	R: P L: P
NIPBL		NM_133433.2	NP_597677.2				
Presumptive mutation (n=1)	1	**c.5378T>G**	**p.Met1793Arg**	**M**	*De novo*	CdLS	R: S/P L: S/P

Variants understood to be novel at the time this manuscript was written are shown in bold typeface. Abbreviations used: M = pathogenic mutation; P = benign polymorphism; VUS = variant of uncertain pathogenicity; MON = Mondini malformation; HL = hearing loss; EVA = enlarged vestibular aqueduct; SCA = semicircular canal abnormalities; UNI = unilateral; C-VCA = cystic vestibulocochlear anomaly; ANH = auditory nerve hypoplasia; IEM = inner ear malformations; MCA = multiple congenital anomalies; USH(#) = Usher syndrome(type); LQT = prolonged QT interval; WS(#) = Waardenburg syndrome(type); PCWH = peripheral demyelinating neuropathy, central dysmyelination, Waardenburg syndrome, Hirschsprung disease; CdLS = Cornelia de Lange syndrome; R = Right; L = Left; Mi = Mild; Mo = Moderate; S = Severe; P = Profound; NL = normal; NA = Not available. *Citations [#] for patients included in cohorts exploring independent research questions. †Atypical phenotype.

As shown in Table 1, 2 patients carried apparently homozygous DNA sequence variations. The parents of the patient with an apparently homozygous mutation in *USH1C* denied consanguinity but are from the same small village. The parents of the patient with 3 apparently homozygous VUS in *CDH23* are first cousins (Table 1).

With the exception of the patient shown in Table 1 to carry 1 mutation and 3 VUS in *OTOF*, benign polymorphisms were not included in this report. An exception was made for the presumptive p.Glu801Leu polymorphism, however, because the phase for this two nucleotide substitution could not be set in this patient, i.e. GA>TT *in cis* versus G>T and A>T *in trans*.

As noted with a dagger symbol in Table 1, atypical phenotypes were observed in 9 patients. Notably, among the 4 patients carrying 2 mutations in *SLC26A4*, 1 patient was found to have asymmetric hearing loss despite having bilateral Mondini malformation, and 2 patients were found to have bilateral malformations of the semicircular canals. Among the 5 patients carrying 1 mutation in *SLC26A4*, 1 patient had unilateral hearing loss with unilateral cystic vestibulocochlear anomaly and auditory nerve hypoplasia on the same side as the hearing loss, 1 patient had unilateral hearing loss and a unilateral enlarged vestibular aqueduct on the same side as the hearing loss, and 1 patient also carried a novel DNA sequence variant of uncertain pathogenicity and had asymmetric hearing loss despite having bilateral Mondini malformation. Among the 2 patients carrying 1 or more variants of uncertain

pathogenicity in *SLC26A4*, 1 patient carrying 2 variants of uncertain pathogenicity had unilateral hearing loss despite having bilateral inner ear malformations involving the vestibule and semicircular canals, and 1 patient carrying a single variant of uncertain pathogenicity had bilateral Mondini malformation with bilateral semicircular canal abnormalities and multiple congenital anomalies of unknown but presumably independent etiology. Additionally, 1 patient with a clinical diagnosis of Usher syndrome type 1 carrying 3 apparently homozygous variants of uncertain pathogenicity in *CDH23* had bilateral malformation of the semicircular canals.

DISCUSSION

Briefly, 37 different DNA sequence variations of known or uncertain pathogenicity were identified in the coding and near-coding regions of 9 genes in 25 of 71 patients with hearing loss whose clinical presentation suggested a genetic syndrome or auditory neuropathy. Of these DNA sequence variations, 14 were understood to be novel at the time this manuscript was written. Atypical phenotypes were observed in 9 patients.

Eight patients with one or more DNA sequence variations in *SLC26A4* and 1 patient with DNA sequence variations in *CDH23* demonstrated additional physical findings not typically thought of as associated with mutations in these genes. In 3 of these patients, 2 mutations in *SLC26A4* were identified. In 6 patients, 5 with variations in *SLC26A4* and 1 with variations in *CDH23*, only 1 mutation or one or more VUS were identified. These observations suggest several possibilites: the phenotypic spectrum associated with mutation in these genes may be broader than typically considered; additional mutations in these genes not detected by the methods used in this study might exist in these patients; mutations in causative or modifier genes not evaluated in this study may be involved; or, environmental factors that modify the phenotypes associated with mutations in these genes might exist.

A definite or presumptive molecular etiology was identified for only 9 of the 71 patients evaluated in this study - 8 suspected of having syndromic hearing loss and 1 with auditory neuropathy. While more extensive sequencing of the regulatory and deep intronic regions of the genes studied might have yielded additional molecular information, the possibility of DNA sequence variations in additional genes or copy number variations must also be considered. Such ambiguities highlight the limitations of traditional gene sequencing approaches that examine only coding and near-coding regions of known causative genes. In contrast, newer technologies such as whole exome and whole genome sequencing,

known as next generation sequencing technologies, allow sequencing of many genes in a single test and in the case of whole genome sequencing permit evalutation of non-coding regions. The more comprehensive genomic coverage of these next generation sequencing technologies support their consideration for the evaluation of patients with highly heterogenous conditions like genetic hearing loss[17-19].

This study illustrates the clinical utility of DNA sequencing in patients whose presentation suggests a genetic syndrome or auditory neuropathy. The failure to identify a genetic etiology in many patients in this study highlights the extreme heterogeneity of genetic hearing loss, the incompleteness of current knowledge of etiologies of hearing loss, and the limitations of conventional DNA sequencing strategies that evaluate only coding and near-coding segments of genes.

ACKNOWLEDGEMENTS

The authors acknowledge the efforts of Dr. Reid Sutton, Dr. Carlos Bacino, Dr. Fernando Scaglia, Dr. Ross Tonini, Claudia Emery and Janet Maxian.

COMPETING INTERESTS

The authors have no competing interests to declare.

FUNDING

This work was supported by the Alkek Foundation for Research, Sid Richardson Foundation, Brown Foundation Research and Bintliff Foundation for Research.

DATA SHARING STATEMENT

The informed consent document for this study was developed prior to contemporary data sharing prescriptions. In compliance with the informed consent document for this study, all relevant data is provided in this article. No additional data will be made available.

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