ORIGINAL ARTICLE

SLC26A4 mutation spectrum associated with DFNB4 deafness and Pendred's syndrome in Pakistanis

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Pendred's syndrome (PDS) is an autosomal-recessive disorder characterized by sensorineural hearing loss and goiter. PDS is caused by mutations of the *SLC26A4* gene encoding pendrin, a transmembrane exchanger of Cl^- , I^- and HCO_3^- , which is expressed in the thyroid and inner ear. *SLC26A4* mutations can also be associated with non-syndromic deafness, DFNB4. The goal of our study was to define the identities and frequencies of *SLC26A4* mutations in 563 large, consanguineous Pakistani families segregating severe-to-profound recessive deafness. Sequence analyses of *SLC26A4* in 46 unreported families segregating deafness linked to *DFNB4/PDS* revealed 16 probable pathogenic variants, 8 of which are novel. The novel variants include three missense substitutions (p.R24L, p.G139V and p.V231M), two splice site mutations (c.304+2T>C and c.1341+3A>C), one frameshift (p.C565MfsX8) and two different genomic deletions affecting exons 1–2 and 11–18. Each of six pathogenic variants (p.V239D, p.Q446R, p.S90L, p.Y556C, p.R24L and p.K715N) was found in more than one family and haplotype analyses suggest that they are founder mutations. Combined with earlier reported data, *SLC26A4* mutations were identified in 56 (7.2%; 95% CI: 5.6–9.2%) of 775 families. Therefore, *SLC26A4* mutations are the most common known cause of genetic deafness in this population. As p.V239D (30%), p.S90L (18%) and p.Q446R (18%) account for approximately two-third of the mutant alleles of *SLC26A4*, hierarchical strategies for mutation detection would be feasible and cost-efficient genetic tests for DFNB4 deafness and PDS in Pakistanis.

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INTRODUCTION

Pendred's syndrome (PDS) (OMIM# 274600) is classically described as bilateral sensorineural hearing loss and thyroid enlargement (goiter). Structural malformations of the inner ear, including enlarged vestibular aqueducts and Mondini defects of the cochlea, are highly penetrant features of PDS.¹ PDS is caused by mutations of the *SLC26A4* gene, which encodes pendrin.² Pendrin is a transmembrane exchanger of Cl^- , I^- and HCO_3^- , which is expressed in the thyroid and inner ear.³ *SLC26A4* mutations have also been implicated in non-syndromic deafness, DFNB4 (OMIM# 600791),⁴ although the genotypic and phenotypic overlap of PDS and DFNB4 has led to uncertainty in their nosology.^{5,6} In 1965, Fraser⁷ phenotypically estimated that PDS accounts for ~10% of hereditary hearing loss. A more recent genotypic survey of different East and South Asian deaf populations showed that *SLC26A4* mutations account for at least 5% of cases of severe-to-profound deafness.⁸ These and other studies collectively suggest that *SLC26A4* mutations are one of the most common causes of genetic deafness in many populations, including Pakistanis.⁸

To date, more than 150 *SLC26A4* mutations have been identified in patients with PDS, DFNB4 or enlarged vestibular aqueduct.⁹ The most prevalent mutations tend to be specific to one or a few related ethnic groups.^{8,10,11} p.T416P and IVS8+1G>A are common in Northern Europe, whereas p.H723R and IVS7-2A>G are the most common in East Asia.^{8,11,12} We earlier identified mutant alleles of *SLC26A4* in 10 (4.7%; 95% CI: 2.6–8.4%) of 212 Pakistani families segregating recessive deafness, which were ascertained through the National Centre of Excellence in Molecular Biology (NCEMB) in Pakistan.⁸ p.S90L was the most commonly detected mutation in that initial study. The different spectra of *SLC26A4* mutations in different ethnic populations should be amenable to ethnically based, hierarchical mutation detection strategies that are rapid and cost-efficient.¹³

The NCEMB study population is a robust model for genotypic studies of non-syndromic and syndromic deafness phenotypes in Pakistan.^{8,14–19} The goal of our study was to define the identities, frequencies and origins of *SLC26A4* pathogenic variants in a much larger cohort of 775 Pakistani families segregating recessive severe-to-profound deafness with or without goiter. We show that *SLC26A4*

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mutations are a common cause of genetic deafness among Pakistanis and their distinctive spectrum lends itself to hierarchical detection strategies.

MATERIALS AND METHODS

Subjects and phenotype analysis

This study was approved by the IRB (International Review Board) at the National Centre of Excellence in Molecular Biology (NCEMB), Lahore, Pakistan (FWA00001758), and the Combined Neuroscience IRB at the National Institutes of Health, USA (OH-93-N-016). Written informed consent was obtained from adult participants and from parents of minor participants of 775 Pakistani families. Hearing was evaluated using pure-tone audiometry at octave frequencies with intensities of up to 100 dBHL. Vestibular function was evaluated by tandem gait and Romberg testing. All family members were examined by a local physician to detect palpable goiter (Supplementary Table 1).

Genotype analysis

Genomic DNA was extracted from 10 ml of peripheral venous blood as described.²⁰ Primers for PCR (polymerase chain reaction) amplification, *SLC26A4* sequencing, single nucleotide polymorphism genotyping and ARMS (amplification refractory mutation system) were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi/). Three fluorescent-labeled microsatellite markers (*D7S2420*, *D7S2459* and *D7S2456*) linked to *SLC26A4* were PCR-amplified and analyzed as described.²⁰ We designed ARMS primers with mismatches to differentially amplify the p.V239D or wild-type alleles. The four primers were PDS6F (wild-type-specific) 5'-GTGCTGGT

CTCACAGCTAAAGATAGA-3', PDS6R (p.V239D-specific) 5'-GTAGTTTTTG GTTGAAACATTGACGA-3', PDS6F (outer) 5'-TTTCCAGAGAGTAGGTTTC TATCTCAGG-3' and PDS6R (outer) 5'-CCAGACTCAGAGAATGAATACAATT GTT-3'. Amplifications comprised 35 step-cycles with an annealing temperature of 58 °C, 50 ng genomic DNA and standard amplification conditions, except that we used a higher concentration (16 µM) of outer primers compared with that of mutant- and wild-type-specific inner primers (8 µM). PCR products were separated by 2% agarose gel electrophoresis and visualized with ethidium bromide.

Statistical analysis

Differences in single nucleotide polymorphism genotype distributions between p.V239D homozygotes and ethnically matched, normal-hearing controls were analyzed using Fisher's exact test.

RESULTS

The study participants comprised 563 families segregating severe-to-profound, prelingual- or congenital-onset, recessive deafness ascertained through the NCEMB after the original 212 families reported by Park *et al.*⁸ We identified 46 additional families co-segregating deafness (Supplementary Table 1) with short tandem repeat markers linked to *SLC26A4*. Sequence analysis of *SLC26A4* revealed 16 probable pathogenic variants co-segregating with deafness in each of the 46 families (Table 1).

Novel variants include missense substitutions, p.R24L (c.71G>T), p.G139V (c.416G>T) and p.V231M (c.694G>A). All of these affect amino acid residues that are conserved among pendrin orthologs (Figure 1). We identified two novel splice site variants, c.304+2T>C and c.1341+3A>C (Table 1), which are predicted to disrupt conserved splice donor site consensus sequences.²¹ We found one frameshift (c.1692_1693insA) predicted to prematurely truncate the protein (p.C565MfsX8). There were two novel genomic deletions of *SLC26A4*: c.-23177_164+1027del24368ins7 deletes exons 1 and 2 and c.1264-477_2090-4927del11202 encompasses exons 11–18. Alu repeats at the breakpoints of c.23177_164+1027del24368ins7 may have led to its origin through unequal crossing over.^{22–25} These novel mutations were not detected in 192 chromosomes from geographically and ethnically matched, normal-hearing participants.

We also identified eight earlier reported pathogenic variants: c.716T>A (p.V239D), c.269C>T (p.S90L), c.1363A>T (p.I455F), c.1667A>G (p.Y556C), c.1337A>G (p.Q446R), c.1115C>T (p.A372V), c.170C>A (p.S57X) and c.2145G>T (p.K715N).^{8,11,26,27} p.V239D, p.S90L, p.Y556C, p.Q446R and p.K715N were detected in more than one family (Tables 1 and 2). p.A372V was detected in compound heterozygosity with c.304+2T>C.

We performed haplotype analyses of linked short tandem repeat markers and single nucleotide polymorphisms (Table 1 and Figure 2) to identify potential founder effects for recurrent variants of *SLC26A4*. The results are consistent with common ancestors for each of the six alleles (Table 2). Although 16 independent p.V239D chromosomes did

Table 1 Mutation spectrum of *SLC26A4* in Pakistani NCEMB families

Nucleotide variation	Effect	Exon	Number of families
c.-23177_c.164+1027del24368ins7	Genomic deletion	1–2	1
c.71G>T	p.R24L	2	2
c.170C>A	p.S57X	3	1
c.269C>T	p.S90L	3	5
c.304+2T>C	p.M103KfsX4 ^a	3	1
c.416G>T	p.G139V	5	1
c.694G>A	p.V231M	6	1
c.716 T>A	p.V239D	6	16
c.1115C>T	p.A372V	9	1
c.1337A>G	p.Q446R	11	10
c.1341+3A>C	p.G439VfsX19 ^a	11	1
c.1363A>T	p.I455F	12	1
c.1667A>G	p.Y556C	15	2
c.1692_1693insA	p.C565MfsX8	15	1
c.1264-477_2090-4927del11202	Genomic deletion	11–18	1
c.2145G>T	p.K715N	19	2

Abbreviation: NCEMB, National Centre of Excellence in Molecular Biology.

^aEffects on protein (GenBank Accession # AAC51873) are predicted by GenScan.

Figure 1 Multiple sequence alignment of pendrin from human, chimpanzee, monkey, dog, rat and mouse. Amino acid residues R24, G139, V231 and K715 of human pendrin are conserved among orthologs. Non-contiguous portions of pendrin are separated by dotted lines.

not share a common *D7S2420/D7S2456* haplotype, the intragenic *SLC26A4* marker (*D7S2459*) allele is the same on all 16 chromosomes. Highly significant associations between p.V239D and each of seven closely linked single nucleotide polymorphisms (Figure 2, Table 3) support the hypothesis that p.V239D is a founder mutation that arose in a common ancestor.

We compared the frequencies of all known mutant alleles of *SLC26A4* (Figure 3) among the NCEMB families. Exons 3, 6 and 11 collectively account for the majority (72%; 41/57) of mutant alleles (Figure 3). As p.V239D accounts for 30% (17/57; 95% CI: 19.5–42.7%) of the mutant alleles, we developed a tetra-primer ARMS assay to rapidly and inexpensively detect p.V239D carriers and homozygotes (Figure 4). The sensitivity and specificity of this assay were confirmed on multiple DNA samples with genotypes established by direct sequencing (Figure 4).

DISCUSSION

Taken together with our earlier result,⁸ *SLC26A4* mutations account for 7.2% (56 of 775 families; 95% CI: 5.6–9.2%, Table 4) of deafness in

the NCEMB Pakistani study population. These results indicate that *SLC26A4* mutations are a significant cause of recessive hearing loss in the Pakistani population (Table 4). Our estimated *SLC26A4* mutation prevalence is not significantly different from that of other studies in different populations. *SLC26A4* mutations account for 8.95% of prelingual deafness in a study of 514 patients from Northern China.¹⁰ In a few eastern and central regions of China, the c.IVS7-2A>G allele alone accounts for 8–12% of individuals with hearing loss.¹² In Koreans and Mongolians, *SLC26A4* mutations account for 6.5% (95% CI: 2.7–14.2) and 2.1% (95% CI: 0.7–5.5) of deafness, respectively.⁸ In Japan, mutations of *GJB2*, *SLC26A4*, *CDH23* and the mitochondrial 12S rRNA are major causes of hearing loss.²⁸ We are yet to evaluate *GJB2* in our NCEMB cohort of deaf participants from Pakistan. However, in another study,²⁹ mutations of *GJB2* also make a significant contribution to hearing loss in Pakistan (Table 4).

Probands of the NCEMB deafness cohort are usually students in schools for the hearing impaired who are often profoundly deaf from birth. This strategy for ascertaining families segregating profound hearing loss will miss families segregating mild or delayed-onset hearing loss. Thus, we have likely overlooked some of the phenotypic variability because of mutations of *SLC26A4* in the Pakistani population and have underestimated the prevalence of DFNB4/PDS. We have also not evaluated participants for enlarged vestibular aqueduct. For economic and geographical reasons, it is not feasible to routinely do a computed tomography scan to detect enlarged vestibular aqueduct. However, PCR-based assays that are used to detect mutations of *SLC26A4* are quick and affordable.

Table 2 *SLC26A4* mutation-linked STR haplotypes

Mutation	STR allele size (bp)			PKDF family code
	<i>D7S2420</i>	<i>D7S2459</i>	<i>D7S2456</i>	
p.Q446R	278	134	239	049, 812
	278	134	247	293, 354, 396, 398, 451, 453, 486, 775
p.V239D	276	140	239	146
	278	140	243	235
	280	140	247	705, 709
	282	140	239	263
	282	140	241	427
	284	140	243	042, 209, 485, 541, 700, 706, 846
	284	140	239	134, 278
	284	140	241	259
p.S90L	276	140	239	347, 726, 734, 831
	276	140	241	376
p.K715N	276	138	241	143
	276	138	239	502
p.R24L	274	138	239	372, 772
p.Y556C	282	138	241	172, 804

Abbreviation: STR, short tandem repeat.

Table 3 SNP analysis of c.716T>A (p.V239D) homozygotes and ethnically matched normal controls

				Number of chromosomes						
				Case (n=16)		Control (n=32)				
dbSNP ID	Position (bp)	Allele 1	Allele 2	1/1	2/2	1/1	2/2	P-value ^a		
rs6949189	107095577	C	G	0	32	32	30	3.0×10 ⁻⁸		
rs2395910	107097048	G	A	0	32	31	25	2.0×10 ⁻⁸		
rs11769313	107101183	G	A	32	0	36	28	8.5×10 ⁻⁷		
rs13232692	107104946	G	A	32	0	26	36	2.0×10 ⁻⁹		
rs10240818	107105651	G	T	0	32	41	23	1.0×10 ⁻¹⁰		
rs6953220	107110427	T	G	0	32	25	37	3.6×10 ⁻⁶		
rs6970857	107110445	C	G	0	32	31	29	3.8×10 ⁻⁸		

Abbreviation: SNP, single nucleotide polymorphism.

^aFisher's exact test.

Figure 2 Physical map of human chromosome 7q22.3. The schematic diagram of *SLC26A4* shows the location of six common mutations, seven single nucleotide polymorphisms (SNPs) and three (short tandem repeat) STR markers used for haplotype analyses. Positions are numbered according to the UCSC genome browser (March 2006 assembly, build 36.1, <http://genome.cse.ucsc.edu/cgi-bin/hgGateway>).

Figure 3 Exonic locations and frequencies of pathogenic *SLC26A4* point mutations in the Pakistani National Centre of Excellence in Molecular Biology (NCEMB) study population, including families from Park *et al.* 2003.⁸ Exons 3, 6 and 11 contain the majority (72.0%) of detected mutations. Besides the 55 point mutations, we detected two deletions (c.-23177_c.164+1027del24368ins7 and c.1264-477_2090-4927del11202) that encompass more than one exon (Table 1).

Figure 4 Results of Tetra Primer amplification refractory mutation system (ARMS) assay for detection of c.716 T>A (p.V239D). Polymerase chain reaction (PCR) amplification using the outer primers generates a 306-bp positive control amplicon in all samples. Nested allele-specific primers generate a 206-bp product from the wild-type allele, whereas the p.V239D allele results in a 151-bp amplicon.

Ten of the mutations of *SLC26A4* identified in this study were private (found in single families). However, six mutations account for more than half of the mutant alleles that we found in Pakistani families (Table 1). We observed a distinctive spectrum of *SLC26A4* mutations that did not include alleles prevalent in other populations, such as c.IVS7-2A>G (Chinese and Koreans), p.H723R (Koreans and Japanese), p.V138F (Germans) or c.766-2A>G (Taiwanese).^{8,30–32} As three alleles (p.S90L, p.V239D and p.Q446R) account for ~66% of the cases of DFNB4/PDS deafness in the NCEMB study population, hierarchical mutation screening would be a cost-effective approach to molecular diagnosis. For example, testing for p.V239D would provide

Table 4 Contribution of different genes to hereditary hearing loss in Pakistani NCEMB cohort of families

Gene	Locus	Percentage (fraction) of Families	95% CI	References
<i>SLC26A4</i>	<i>DFNB4/PDS</i>	7.2 (56/775)	5.6–9.2	This study; Park <i>et al.</i> ⁸
<i>GJB2</i>	<i>DFNB1</i>	6.1 (12/196)	3.5–10.3	Santos <i>et al.</i> ²⁹
<i>TMC1</i>	<i>DFNB7/11</i>	3.4 (19/557)	2.2–5.3	Chishti <i>et al.</i> ¹⁵
<i>MYO15A</i>	<i>DFNB3</i>	3.3 (20/600)	2.2–5.1	Nal <i>et al.</i> ³³
<i>OTOF</i>	<i>DFNB9</i>	2.3 (13/557)	1.4–4.0	Choi <i>et al.</i> ³⁴
<i>TRIOBP</i>	<i>DFNB28</i>	1.6 (9/557)	0.8–3.0	Riazuddin <i>et al.</i> ¹⁸
<i>TRIC</i>	<i>DFNB49</i>	1.1 (3/284)	0.3–3.0	Chishti <i>et al.</i> ¹⁵ Riazuddin <i>et al.</i> ¹⁷
		1.4 (8/557)	0.7–2.8	
<i>MYO6</i>	<i>DFNB37</i>	1.2 (3/250)	0.4–3.4	Ahmed <i>et al.</i> ³⁵
<i>RDX</i>	<i>DFNB24</i>	0.3 (2/557)	0.1–1.3	Khan <i>et al.</i> ³⁶

Abbreviations: CI, confidence interval; NCEMB, National Centre of Excellence in Molecular Biology.

a definitive result for ~30% of Pakistani patients with DFNB4/PDS. Our tetra-primer ARMS assay for p.V239D would be a logical first step in a strategy that includes similar assays for other prevalent alleles, such as p.S90L and p.Q446R. As the majority of NCEMB families are from the Punjab province and speak Punjabi,¹⁶ the details of a hierarchical *SLC26A4* mutation screening strategy may need to be refined for other geographical regions and lingo-ethnic groups within Pakistan.

ELECTRONIC DATABASE INFORMATION

Primer3; http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi/
GenScan server at MIT; <http://genes.mit.edu/GENSCAN.html>.

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