

Research paper

Prevalence of two mutations p.V239D and p.Q446R of *slc26a4* gene in hearing impaired individuals in Khyber Pakhtunkhwa, Pakistan

Karim Ruknuddin¹, Tahira Mohammad Ghulam¹, Bilal Ahmed Mian²,
Muhammad Ilyas³, Khitab Gul*¹

¹Department of Biosciences, Mohammad Ali Jinnah University, Karachi, Pakistan

²Centre for Human Genetics, Hazara University, Mansehra, Pakistan

³Centre for Omics Sciences Islamia College University Peshawar, Pakistan

*Corresponding Author's Email: drkhitabgul@jinnah.edu

Abstract

Deafness is the most prevalent sensory disorder affecting 1-3.47 in 1000 live births. Approximately 15% of world's population are affected by some degree of hearing loss, in which 17% have severe hearing impairment. Genetic hearing loss accounts for 50% of hearing loss from which 70% are from recessive trait in non-syndromic hearing loss (NSHL). Earlier epidemiological studies indicated mutations in *SLC26A4* gene is one of the predominantly genes involved in ARNSHL is associated with enlarged vestibular aqueduct (EVA). The autosomal recessive non-syndromic hearing loss (ARNSHL) is associated with mutations in solute carrier family 26, member 4 (A4) gene which is the among most common known causes of ARNSHL worldwide. Over 200 mutations have been identified in *SLC26A4* gene. Different regions and countries have identified specific variants of *SLC26A4* gene, among which p.V239D and p.Q446R are more frequent mutation in Pakistani Punjabi population. Studies have showed difference of frequency distribution of *SLC26A4* linked to hearing loss among different ethnicities of Pakistan. This study is aimed to determine the frequency of two variants p.V239D and p.Q446R of *SLC26A4* gene in Peshawar, KPK. All 26 individuals voluntarily participated in this study, after taken informed consent from patients or their guardians, peripheral blood samples were collected. DNA was extracted from these samples followed by DNA quantification and quality assessment. Tetra-primer Amplification Refractory Mutation System (T-ARMS) PCR was successfully applied on 26 samples. All findings were compared with normal individuals control. It will be very beneficial to find variants of *SLC26A4* and other genes responsible for hearing loss in different ethnic groups of Pakistan. This study will help to provide a cost effective methods for genotyping point mutations associated with hearing loss.

Keywords: Hearing loss, *SLC26A4*, non-syndromic hearing loss, ARNSHL, ARMS-PCR

Introduction

Hearing loss (HL) is a common disability, defined by impaired ability to detect sound frequency in normal range. Hearing loss has four categories mild, moderate, severe and

profound hearing loss, where, severe and profound hearing loss refers to as total lack of sensitivity or deafness which affects day to day communication and quality of life (1, 2).

According to World Health Organization statistics of 2018, Approximately, there are 466 million people (6.1% of world population) with hearing disability. Hearing loss negatively impacts on individual's quality of life. It causes problems in day to day communication with impact significantly on cognitive, social and emotional and physical wellbeing of affected individual's health (3). Hearing impairment is a lifelong disease and considered most prevalent sensory defect in humans worldwide. Hearing loss affects language acquisition, speech development and proper education in children which often results in less opportunities for work which leads people with deafness to isolate from society (4).

Different criteria had been classified for hearing loss like pre-lingual or post-lingual, genetic or environmental, syndromic or non-syndromic types of hearing loss (5). Pre-lingual hearing loss can be either congenital or delayed on-set, it occurs before the development of language and speech, usually just before the age of 2 years (6). Approximately 30% of hearing loss which results from genetic cause are syndromic (7) in which Waardenburg, Pendred and Usher are well known, associated with genetic hearing loss (8). Genetic factors of hearing loss accounts for 50% to 70%. (5, 9). The prevalence in infants and newborns are 1 to 3.47 cases in every 1000 live births and it is the most common form of sensorineural disorder in humans and the most frequent birth defect in developed societies (10).

Pakistan's estimated rate of severe of hearing impairment rate in live birth is higher due consanguineous families from which 40% are among first cousin marriages (11, 12). Non-syndromic hearing loss has been further sub-categorized into autosomal recessive (75% to 80%), autosomal dominant (20%), X-linked, mitochondrial, miRNA (<1%) (13).

Genes of non-syndromic hearing loss are mapped at different locations at chromosomes which are arranged in their pattern of discovery and are designated specific term for deafness known as DFN (Deafness). Sub-categories like autosomal dominant loci are known as DFNA (Deafness Autosomal), autosomal recessive loci its DFNB (Deafness recessive) and X-linked (DFN) loci are referred as DFN. Linkage analysis have mapped many genes of non-syndromic hearing loss to different chromosomes, among which are most frequent driver mutation for monogenic hearing loss is the connexin-26 gene referred to as *DFNB1*. Autosomal dominant hearing loss mutation in *DFNA9* (COCH gene), *DFN3* (POU3F4 gene) and certain X-linked loci (COL4A6) are associated with post-lingual hearing loss. Small mutation of 12S rRNA is responsible for mitochondrial hearing loss (14).

There are 34 variants (Table 1) known to be associated with SLC26A4 causing autosomal recessive non syndromic hearing loss DFNB4(https://www.uniprot.org/uniprot/O43511#pathology_and_biotech). The aim of this study was to find the prevalence of two most common variations p.V239D and p.Q446R of SLC26A4 in Peshawar, Khyber Pakhtunkwa, Pakistan.

Material and Methods

Total 26 hearing impaired individuals were included in the study by visiting different special schools.

Sample Collection

Patients belonged to Pashtun ethnic background and were collected from Peshawar KPK province of Pakistan. They were isolated cases with hearing disability.

No.	dbSNP id	Amino Acid Change
1	rs539699299	S28R
2	rs539699299	S90L
3	rs145254330	L117F
4	rs984967571	P123S
5	rs1554354370	T132I
6	rs760413427	M147V
7	rs760413427	R185T
8	rs111033303	G209V
9		A227P
10	rs80338848	L236P
11	rs111033256	V239D
12	rs1315422549	S252P
13	rs727505080	V281I
14	rs111033212	F335L
15	rs121908361	K369E
16	rs121908364	A372V
17	rs201562855	N392Y
18	rs397516414	V402M
19	rs111033305	R409P
20	rs111033220	T410M
21	rs28939086	T416P
22	rs111033307	L445W
23	rs768471577	Q446R
24	rs1554359670	N457K
25	rs200511789	I490L
26	rs111033308	G497S
27	rs747636919	Y530S
28	rs763006761	Y556C
29		N558K
30	rs1584337274	S666F
31	rs111033318	L676Q
32	rs121908363	T721M
33	rs121908362	H723R
34	rs1562845849	M775T

Table 1: Known Variants of SLC26A4 associated with DFNB4 in dbSNP database.

Hearing impairment was confirmed via pure tone audiometry. Physical assessment was conducted in all selected individuals, patients with any syndromic phenotype or any other comorbidities identified via clinical assessment were rule out, deafness in individuals due to any inflammatory conditions or environmental factors were excluded from this study. Legal guardian of each individual was informed in depth about the study and informed consents were obtained from them. This study was approved by research committee of Mohammad Ali Jinnah University.

Blood sampling and DNA extraction

Blood sample (5ml) is drawn from patient in tube containing anti-coagulant i.e. EDTA (Ethylene Diamine Tetra Acetic Acid). Samples are stored at -20°C until processed for downstream application. DNA extraction is proceeded by removing EDTA tubes from -20°C and thawed them at room temperature for 30 minutes, then: White blood cells (WBCs) are separated from red blood cells (RBCs) by adding three volume of red cell lysis buffer (RCLB). For thorough mixing, tube was inverted and vortexed for at least 5 minutes and then centrifuged (Hitachi) at 20,000 g for 10 minutes. Supernatant was decanted while pellet was re-suspended again in (RCLB) and repeated step number 2 and 3 until clear pellet was observed. After getting clean appearance, pellet was re-suspended with equal volume of cell lysis buffer and 10µl of Proteinase K (10mg/ml) (ThermoFisher). Sample were kept in water bath (Thermoscientific) at 56°C for about 1 hour. Phenol was added in equal volume and mixed well followed by centrifugation at 10,000 g at 4°C for 10 minutes. Upper

aqueous layer was transferred in a new tube containing equal volumes (1:1) of phenol and chloroform: isoamylalcohol (24:1) and mixed thoroughly. Sample was centrifuged at 4°C for 10 minutes at 10,000 g. The supernatant was then transferred to a fresh tube and supernatant was aspirated in a fresh tube. Two volume of absolute alcohol was added with supernatant, mixed well with subsequent centrifugation 10,000 g (4°C) for 20 min. 70% Ethanol was added with pellet after removing supernatant followed by centrifugation at 10,000 g for 10 minutes and aspirated the supernatant without disturbing the pellet. The pellet then air dried and finally re-suspended in TE (Tris EDTA) buffer and stored at -20°C.

DNA Quantification:

Nano drop (2000C, Thermo scientific) was used to measure the Optical Density (O.D) of extracted DNA. DNA concentration and 260/280 ratio sample was then read at 260/280nm nm to measure DNA concentration. All the samples between purity of 1.7 to 2.0 were taken forward in this study.

Primer Designing

ARMS PCR tetra-primer were developed for genotyping p.V239D (c.716 T>A) and p.Q446R (c.1337 A>G) mutations in *SLC26A4* gene. The primers were designed by using Primer1 server (<http://primer1.soton.ac.uk/primer1.html>) for PCR oligonucleotides designing. The primers were designed as per standard criteria for ARMS PCR. The point mutations are positioned asymmetrically with respect to common outer primer, so that different allele specific amplicon product lengths could be

easily separated by agarose gel electrophoresis. The specificity of the primers was verified by “BLAST” program at <http://www.ncbi.nlm.nih.gov/blast>. Primers were ordered from Bio Basic Canada Info. Primers were received in lyophilized form, and were reconstituted in Tris EDTA (TE) buffer to make 100µM stock solution and working aliquots of 10µm were prepared and stored stock solution at -20 °C before use. Primers used in this study are listed in table 2.

Genotype	Primer sequence	Product size	Annealing Temp °C
p.V239D (c.716 T>A)	Forward inner primer (T allele) AGTGCTGGTCTCACAGCTAA AGATCGT Reverse inner primer (A allele) CCATTGTAGTTTTTGGTTGAA ACATTGAAAGT Forward outer primer TCCAGAGAGTAGGTTTCTATC TCAGGCAA Reverse outer primer GAGATGAGGTCTCACGTCTC AAACTCC	Control Fragment: 402 bp T allele:251 bp A allele: 209 bp	63°C
p.Q446R (c.1337 A>G)	Forward inner primer (G allele): CTGGGGAAGCTTCTGGAACC CTTTCG Reverse inner primer (A Allele) CGGTATGCAGAGAAGCAGGG TTATACCTGCT Forward outer primer TCCAGTGAGCTGGAAGACAC AAGGGAGA Forward outer primer TCTCCACAGACGAGGAATGT CACACAGC	Control Fragment: 362 bp G allele: 249 bp A allele: 170 bp	64°C

Table 2: Sequences of primers used, annealing temperature and product size for ARMS-PCR of SLC26A4 gene.

Amplification Refractory Mutation System or ARMS-PCR:

SNP detection via PCR is usually done via ARMS-PCR technique. Two different sets of tetra-primers for each SNP, outer primers are used as internal control which assess the overall PCR reaction and integrity of DNA, whereas inner primers were specific to a particular allele. The inner primers were

designed to target SNP of interest and had of different product length. PCR was run using Dragon Lab® Gradient PCR thermal cycler. For each SNP, PCR conditions were separately optimized. Total of 10µl PCR mix was prepared for amplification and entire product was load in agarose gel. Target SNPs were c.716 T>A (V239D) and c.1337 A>G (Q446R). 100 ng of DNA was used from each sample for ARMS-PCR.

PCR Mix Preparation:

Master Mix was prepared of each tetra-primer in order to screen patient samples. All reagents concentration of both tetra- primer were same tetra-primer concentration. The tetra-primer concentration of p.V239D SNP c.716 T>A was 1:0.6 ratio of outer primers to inner primer, and for tetra-primer of Q(446)R SNP c.1337 A>G, the ratio was 1:1.2 in outer primer to inner primer ratio. Other reagents included 100ng DNA of each sample added separately with mix containing MgCl₂ 3mM, dNTPs 0.4mM, taq polymerase 1.875 units, 1x taq polymerase buffer, 0.4% DMSO and DdH₂O as needed to top-up the total volume to 10µl in each PCR tube.

Primers were ordered from Bio Basic Canada Inc. All other reagents for PCR were purchase from Promega, USA brand.

PCR Conditions:

Both tetra-primers for ARMS-PCR were optimized at slightly different PCR. The difference was in annealing temperature and PCR cycles. The PCR p.V239D and p.Q446R the conditions were as follows: first step- Initial denaturation at 95°C for 3 minutes, second step- denaturation at 95°C for 1 minutes, third step- annealing at 63°C for 45 seconds for p.V239D (c.716T>A) and 64°C

for 1 minute for p.Q446R (c.1337A>G) with 25 cycles and 30 cycles respectively, followed by extension at 72°C for 1 minutes, and final extension for 10 minutes at 72°C. At the final step incubation at 4°C until products were separated on agarose gel electrophoresis.

Agarose Gel Electrophoresis:

2.5gm of agarose weighed in a weighing paper and added in 100ml of 1_xTAE buffer. Flask containing agarose and buffer were microwaved at 750 volts until agarose is completely dissolved.

Flask was cool down till 50°C and then 0.2-0.5µg/ml (around 2-3µl) of ethidium bromide. Dissolved agarose gel is poured in Bio-Rad gel casting tray and comb is placed appropriately on the tray as it does not touch the bottom.

The PCR products were then loaded into the wells of 2.5% agarose gel for fragment. Separation in Bio-Rad Electrophoresis apparatus. Generally, 2.5% gels separate 100-1000bp DNA fragment easily. 5µl of 100 base pairs ladder (MOLEQULE-ON) was loaded in order to compare and confirm target band size. 1_x TAE running buffer is used to submerged the gel in the gel electrophoresis assembly tank. PCR products were ran via gel electrophoresis at 100 volts for 50 minutes until the bands were properly separated.

All gels were visualized on Bio-Rad Gel-Doc system. The bands appeared bright in contrast with the gel background. Gel-Doc system visualizes DNA bands with presence of ethidium bromide via UV. Ethidium Bromide intercalates with DNA via its complementary bases.

Results

A total of 26 isolated patients from Peshawar city Khyber Pakhtunkhwa, Pakistan were screened for two known mutations of *SLC26A4* gene i.e. p.V239D and p.Q446R. These two mutations have been reported previously in Pakistani population (15) but interestingly, they were absent in our cohort of population.

Figure 1: Gel electrophoresis results of Tetra primers c.716 T>A SNP (V239D) Gel image is of DNA samples amplified through ARMS-PCR. M= 100bp ladder, (a) lane labelled 1-13 and in (b) lane labelled 14-26 are amplified products of patient's samples; lane labelled N is wild type in both gel images. Tetra-primers of c.716 T>A SNP are used for PCR amplification has different product size if homozygous mutated=402/209bp (A/A), heterozygous carrier=402/251/209 (T/A), homozygous wild type=402/251bp (T/T).

Figure 1A: Image of p.V239D SNP c.716 T>A PCR products separated on agarose gel.

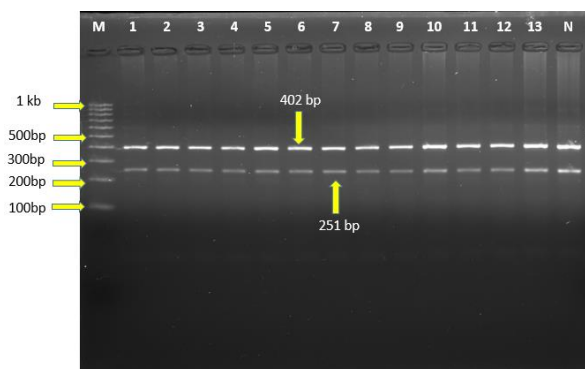


Figure 1B: Image of p.V239D SNP c.716 T>A PCR products separated on agarose gel.

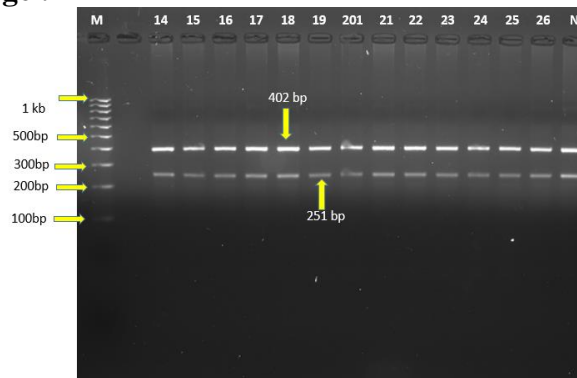


Figure 2: Gel electrophoresis results of Tetra primers of c.1337 A>G (Q446R). Gel image of DNA samples amplified through ARMS-PCR. M= 100bp ladder; (a) lane labelled 1-13 and in (b) lane labelled 14-26 are amplified products of patient's samples; lane labelled N is wild type in both images. Tetra-primers of c.1337 A>G SNP are used for PCR amplification has different product size if homozygous mutated=362/249bp (G/G), heterozygous carrier=362/249/170bp (A/G), homozygous wild type=362/170bp (A/A).

Figure 2A: Image of p.Q446R SNP c.1337 A>G PCR products separated on agarose gel:

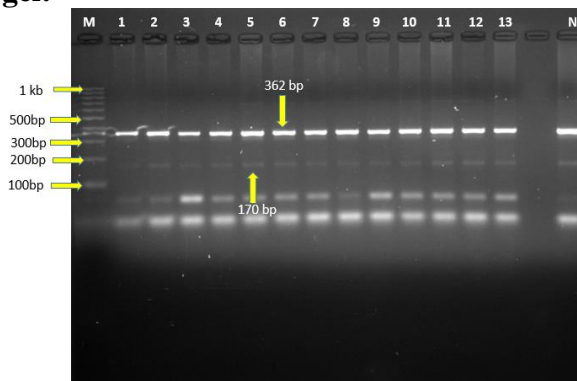
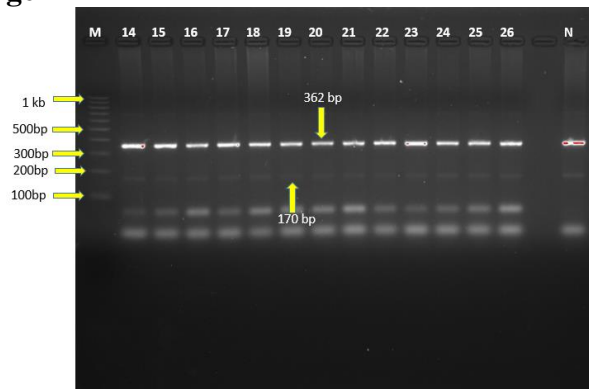


Figure 2B: Image of p.Q446R SNP c.1337 A>G PCR products separated on agarose gel



Discussion

The prevalence of recessive mutations varies among different ethnic groups (16). Mutations in *SLC26A4* gene such as p.V138F, p.T416P, p.L236P, p.L445W are commonly observed in Middle Eastern, European, North African, and North Eurasians whereas p.H723R and p.V239D mutations observed in South Asian, East Asian and North East Asian (17). Recent study reveals that deaf population of East and South Asia accounts for 5% for cases with severe to profound hearing loss (16). The most prevalent genes which carry mutations in patients include *MYO15A*, *OTOF*, *CDH23*, *TMCI*, *GJB2* and *SLC26A4* (18). In different regions and countries, Specific variants of *SLC26A4* have been reported. In Northern European population, c.1001+1 G>A and p.T416P were among the most frequently identified mutations in *SLC26A4* (19), the frequency of p.H723R variant of *SLC26A4* gene is 1.39% to the normal hearing population of Asia (20). Other study which

shows that mutations in *SLC26A4* gene is one of the common causes of genetic hearing loss among Pakistani population (15).

In the current study, no mutations for both variants, p.V239D and p.Q446R of *SLC26A4* gene were found in our study samples. Several investigators have noticed that many patients lack mutation in either single or both alleles of *SLC26A4* (21, 22).

It is possible that genes other than *SLC26A4* are responsible for hearing loss in this studied group. In a study conducted by Yang et al, patients with hearing impairment and EVA, in which thyroid criteria was not rule out, 19% of sibling had mutation in one allele and 42% children did not have any mutation in *SLC26A4* gene, suggesting that involvement of other genetic factors (23).

Conclusion

These two known SNPs of *SLC26A4* gene i.e. p.V239D and p.Q446R were not detected in our study samples. The possible reasons behind this could be the small number of cases included in this study, genetic load biasness of other ethnic groups in the population, or very high rate of consanguineous marriages. These samples were mainly from KPK province; it is a possibility that the frequency of these variants is much lower in Peshawar, KPK than previously reported in Pakistani Punjabi population. There is also a possibility that other genes or other mutations of *SLC26A4* might be responsible for hearing loss in this ethnic group. Screening of large cohort for these variants may provide the frequency in this ethnic group.

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