Universitat Autònoma de Barcelona

THE IDENTIFICATION OF THE GENETIC DEFECTS UNDERLYING MONOGENIC RECESSIVE HEARING LOSS AT ARAB POPULATION

Walaa Mohamed

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THE IDENTIFICATION OF THE GENETIC DEFECTS UNDERLYING MONOGENIC RECESSIVE HEARING LOSS AT ARAB POPULATION

Ph.D. Thesis
In Genetics

DEPARTAMENT DE GENÈTICA I MICROBIOLOGIA
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Report presented by **Walaa Mohamed** in order to complete the requirements to be granted the degree of Doctor of Philosophy in Genetics by the Autonomous University of Barcelona.

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In the name of God, the Gracious, the Merciful.

Recite in the name of your Lord who created -

Created man from a clinging substance.

Recite, and your Lord is the most Generous -

Who taught by the pen -

Taught man that which he knew not.

No! [But] indeed, man transgresses-

Because he sees himself self-sufficient.

Sūrat Iqrā

Juz' 30

أهدي هذا النجاح لابنتي الجميلة، والدتي ووالدي. أطال الله في أعمارهم.

To my beautiful daughter (Fatima), my wonderful mother and father.

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ABBREVIATIONS

12s rRNA	12S ribosomal RNA
ATP	Adenosine triphosphate
ARNSHL	Autosomal recessive non-syndromic hearing loss
CLDN14	Claudin-14
CES	Clinical Exome Sequencing
Cx26	Connexin 26
Cx30	Connexin 30
Cx31	Connexin31
dB	Decibels
D-loop	Displacement loop
GJB2	Gap junction protein beta 2
GJB4	Gap junction protein beta 4
GJB6	Gap junction protein beta 6
HL	Hearing loss
mtDNA	Mitochondrial DNA
NGS	Next-generation sequencing
NSAHL	Non-syndromic autosomal recessive hearing loss
SNHL	Sensorineural hearing loss
SNPs	Single-nucleotide polymorphisms
TMC1	Transmembrane channel-like protein
WES	Whole Exome Sequencing

ABSTRACT

Congenital genetic disorders are important at all healthcare levels due to their significant burden on affected individuals and societies. They may be caused by genetic factors or be triggered by environmental exposures. A high prevalence of consanguineous marriages is present in many communities worldwide, especially countries of the Middle East and North Africa. Due to high consanguinity rates in these populations, there is an increased rate of congenital monogenic disorders, including non-syndromic autosomal recessive hearing loss (NSAHL). The NSAHL condition accounts for many birth defects and disabilities among live births in UAE and Arabs. Therefore, unraveling the genetic causes of NSAHLs is of great value for families and society as a whole. These findings will lead to the development of accurate molecular screening tests and improving genetic counseling. The recent advances in technologies such as microsatellite markers, clinical-exome sequencing, and whole-exome sequencing revolutionized genetic research. They have revealed the genetic defects of many monogenic conditions and the co-segregation among the family probands. Therefore, we aim to identify the genetic causes of NSAHL underlying a broad spectrum of congenital conditions in the Arab population. Patients were recruited and clinically evaluated by an expert geneticist. The main approaches were : (i) localize the homozygous regions in each particular family using homozygosity mapping, (ii) sequence the whole- and clinical exome of affected individuals using next-generation sequencing platform to reveal all variants including the pathogenic mutations, finally (iii) perform functional assays to validate or rule out the pathogenicity of the identified variants. We found that the inherited HL was high in the Arab population due to consanguineous marriage. *GJB2*, *CLDN14* and *TMC1* genes found to be related with HL among the Arab populations. Our findings provide new genetic markers for genetic counseling, prenatal and postnatal diagnosis of HL in the Arab population.

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1. INTRODUCTION

Hereditary hearing loss (HL) is one of the most common birth defects, with an approximate incidence of 1–2 per 1,000 newborns presenting bilateral sensorineural HL at the time of newborn hearing screening. In developed countries, HL stems from both environmental and genetic etiological factors, with the genetic contribution comprising 50–60% of cases [1,2].

The quest for new genes, especially in the last decade, has been profoundly successful due to the Mendelian existence of non-syndromic HL (NSHL). NSHL exhibits extreme genetic heterogeneity, with more than 73 autosomal dominant (deafness, neurosensory, autosomal dominant (DFNA)), 108 autosomal recessive (deafness, neurosensory, autosomal recessive (DFNB)) and 6 X-linked (deafness, neurosensory, X-linked (DFNX)) loci with 48, 76, and 5 causative genes, respectively, identified to date (http:/hereditaryhearingloss.org). A fraction of these genes has been associated with both dominant and recessive HL. Besides, mitochondrial mutations may also underlie NSHL. For decades, homozygosity mapping has played an important role in gene discovery by detecting the homozygous regions followed by screening for recessive mutations using families of consanguineous marriages [3].

Next-generation sequencing (NGS) technologies are causing a shift in how clinical geneticists and medical researchers investigate genetic disorders [1,2] and provide powerful application not only to molecular diagnostics but also to the discovery of new genes and further characterization of already-known disease-associated genes [1,3,4]. Of particular interest to clinicians is target capture NGS involving a subset of disease-relevant genes in the form of gene panels that accommodate sequencing of dozens or hundreds of genes in parallel, with a clear advantage over conventional polymerase chain reaction-based Sanger sequencing approaches by achieving faster results at a fraction of the cost [5]. A further application of NGS is learning the varied landscape of the minor allele load on a gene-by-gene, exome-wide, or genome-wide basis in affected and unaffected individuals. Understanding the concept of mutational load in human disorders will provide insight into the potential role of rare non-synonymous single-nucleotide polymorphisms (SNPs), their maintenance throughout human evolution, and their predication underlying human disease. By shifting emphasis away from individual frequencies of deleterious variants toward cumulative frequencies, explanations for common disorders with complex

inheritance become plausible [6–8]. Therefore, we aimed to apply these recent technologies to extend the study to newly diagnosed NSAHL in inbred families from Arabs.

1.1 STRUCTURE OF THE EAR AND THE HEARING PROCESS

The ear is a very efficient transducer (i.e., a device that changes energy from one form to another), changing sound pressure in the air into a neural-electrical signal that is translated by the brain as speech, music, noise, etc. [9]. The human ear is the organ of hearing and balance, it has the ability to convert sound waves into signals that can be transmitted to the brain via nerves to be recognized [10]. The structure of the ear is divided into three parts: the outer ear, the middle ear, and the inner ear **(Figure 1)**. The outer ear is where the sound first pass through a channel which will lead it to further into the middle and inner ear, the outer ear accepts external sounds and will cause the eardrum to vibrate along the ear canal. The vibration of the tympanic membrane of the middle ear causes three small bones-cone bone, stirrup and drill bone to vibrate, transmitting the sound to the inner ear. The inner ear can produce nerve impulses, which are converted into nerve energy along the auditory nerve, and the sound information is transmitted to the brain from there [11].

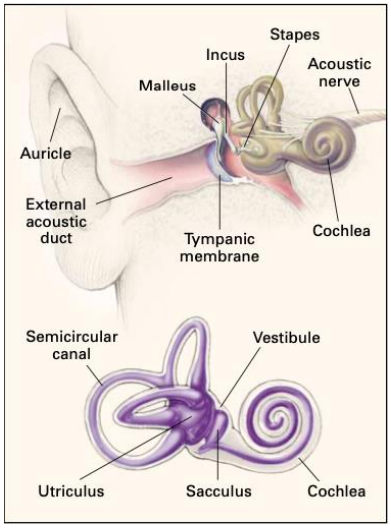

FIGURE 1. DRAWING OF THE OUTER, INNER AND MIDDLE EARS. Sound waves are captured by the auricle and transmitted through the external acoustic duct to the tympanic membrane, which causes the membrane to vibrate. These vibrations are transmitted through the auditory ossicles of the middle ear to the footplate of the staples, which is anchored in the oval window of the cochlea-vestibule. Figure from [12]

To explain briefly the process of hearing is when the sound is emitted, the surrounding air molecules vibrate in a series of vibrations. These vibrations are called sound waves. When the sound waves reach the outer ear, the sound is introduced into the external auditory meatus and reaches the eardrum through the collection of the pinna. The tympanic membrane is the dividing line between the outer and middle ears. It is as thin as paper, but very strong. When sound waves hit the eardrum, it causes vibration of the eardrum. In the middle ear cavity behind the tympanic membrane, there are three interconnected ossicles. Each ossicle is only the size of a grain of rice, which is the smallest bone in the human body. When sound waves vibrate the eardrum, the ossicles also vibrate. The three ossicles form a lever system that amplifies and transmits the sound into the inner ear. In the inner ear, there is an organ specializing in the hearing process, the cochlea. The cochlea has a channel that leads it to the organ of Corti, there are thousands of hair cells in the cochlea, which convert sound signals into bioelectrical signals and transmit them to the brain via the auditory nerve. The brain then processes and integrates the delivered information to produce hearing [9–11].

Further, the inner ear contains a very important organ-semicircular canal. The semicircular canal is composed of three mutually perpendicular small rings, which specializes in the sense of balance in the three-dimensional space of the head. When there is something wrong with the semicircular canal, dizziness may occur [11].

1.2 HEARING LOSS

Hearing loss is a common sensory impairment that affects 278 million people worldwide [13]. HL is also called hearing impairment. It is the number of decibels (dB) above the normal hearing threshold (-10 to 15 dB) of the human ear at a certain frequency. There are three types of HL: conductive, sensorineural and mixed HL. Conductive hearing loss happens when the vibration cannot pass from outer to the inner ear especially through the cochlea. It is caused by malfunction of the ossicles, defect in the eardrum, excessive earwax and finally due to ear infection with inflammation and fluid buildup. The second type sensorineural HL is caused by a dysfunction of the inner ear, the cochlea and auditory nerve, or brain damage. It can also be due to damaged hair cells in the cochlea which might be related to age or because of long term exposure to loud noises,

especially high-frequency sounds. Sensorineural HL is difficult to cure due to the location and complex morphology of inner ears [11,14]. It is a clinically heterogeneous disease leading to negative impacts on quality of life (QOL) in all generations. Sensorineural hearing loss involves different onset, severity and pathological sites [15].The third type of HL is mixed HL, which is a combination of conductive and sensorineural hearing loss.

When hearing loss is suspected, there are hearing screening tests which varies depending on age. For infants, there are two types of tests which are painless and can be done while sleeping. These are otoacoustic emissions and auditory brainstem response [16]. A pure-tone test is used to evaluate hearing deficits by spot-checking certain frequencies, or to evaluate deficits more completely for adults [17]. Pure-tone audiometry is performed with the use of an audiometer. Handheld audiometers have a sensitivity of 92 percent and a specificity of 94 percent in detecting sensorineural hearing impairment [18]. There are four levels of deafness or hearing impairment- mild deafness or mild hearing impairment, moderate deafness or moderate hearing impairment, severe deafness and profound deafness. For the mild deafness, the individual can only detect sounds between 25 and 29 dB and they might find it hard to understand the words other people are saying, especially if there is a lot of background noise. In moderate deafness, individual can only detect sounds between 40 and 69 dB and they need a hearing aid in order to communicate. For the severe deafness, individuals can only hear sounds above 70 to 89 dB and a severely deaf individual can communicate only by lip-read or use sign language even if they have a hearing aid. Finally, individuals who cannot hear a sound below 90 dB has profound deafness. Some people with profound deafness cannot hear anything at all, at any decibel level. Communication is carried out using sign language, lip-reading or reading and writing [19,20].

Deafness or HL is a major problem in the field of occupational and environmental health and can be caused by genetic or environmental factors or a combination of both [15,21].

1.2.1 Environmental factors causing hearing loss

Exposure to noise is recognized as one of the major environmental factors causing hearing loss [14], It can injure the inner ear and produce a sensorineural HL. The HL from noise can be temporary or permanent. Since World War II, considerable effort has been given to studies of noise-induced HL [22]. Enormous noise may lead to either mechanical or metabolic cochlear damage. Mechanical damage to cochlear structures is dominated at a very high intensity of noise [23,24], whereas metabolic cochlear damage dominates at lower noise levels. Probably, free radicals and other reactive endogenous substances play a role in this process [25,26].

After a lifetime of noise exposure, it is difficult to distinguish between noise induced HL and age-related HL in terms of audiometrically as well as histologically. Although models have been developed to predict the amount of hearing loss on the basis of the total amount of noise a worker is exposed to [27,28], susceptibility for noise-induced hearing loss seems to vary widely. It is possible that some individuals are more sensitive to noise due to less effective protective strategies of the middle and inner ear or due to a higher vulnerability of the hair cells [29].

Aside from noise, another possible cause of HL is the use of ototoxic medication, especially in the elderly as they often use more medication than other age groups. Since they have altered renal and liver functions, medication can cause blood levels to rise above critical levels [30]. Aminoglycoside antibiotics can damage hair cells in the same pattern as noise does, also causing a non-reversible hearing loss predominantly in the high frequencies. Aminoglycosides seem to potentiate the ototoxic effect of noise and vice versa [31]. Other medications with potential ototoxic effects have also been proposed, such as nonsteroidal anti-inflammatory drugs (ibuprofen and naproxen), aspirin (when used in large doses; 8 to 12 pills a day), loop diuretics used to treat high blood pressure and heart failure, such as furosemide (Lasix) or bumetanide, and medicines used to treat cancer, including cyclophosphamide, cisplatin, and bleomycin. [32,33].

The effect of tobacco smoking on hearing loss is controversial. Some authors reported that smoking can cause hearing loss [34,35], whereas other studies could not demonstrate smoking to be a risk factor [36–38]. An association between clear alcohol abuse and hearing loss could be demonstrated [34] but there seems to be no relationship between hearing thresholds and moderate intake of alcohol [36].

1.2.2 GENETICS FACTORS CAUSING HEARING LOSS

Genetic factors make some people more susceptible to HL than others. Genetics HL is hereditary and is caused by gene mutations. Hereditary hearing loss and deafness can be conductive, sensorineural, or a combination of both. It can also be either syndromic or nonsyndromic. Syndromic is correlated with the impairments of the external ear or other organs as well malformation of other organs caused by medical problems while nonsyndromic has no visible correlation with any abnormalities of the external ear or any related medical problems. The onset of hearing loss can happen before (pre-lingual) or after (post-lingual) language development [29]. As it is similar in many disorders, syndromic hearing loss pathology varies widely; whereas, in nonsyndromic hearing loss (NSHL), the defect is generally sensorineural and the majority of the cases (70%) is non-syndromic [30]. NSHL can be classified by the mode of inheritance; autosomal dominant (~15- 20%), autosomal recessive (~80%), X-linked, Y-linked and mitochondrial (together ~2%) [39–41]. NSHL is known by its extremely genetic heterogeneity. To date, more than 6,000 causative variants have been identified in more than 110 genes. Multigene panel was used for the largest comprehensive genetics testing, the study involved 440 individuals and it could identify more than 40 causative genes [42]. This extreme genetic heterogeneity highlight the importance of using multigene advance sequencing panels to ease the genetic diagnosis [43].

The autosomal dominant nonsyndromic hearing loss is typically associated with post-lingual, progressive hearing loss that can range from mild to profound at onset or over the course of many decades [44]. To date, there are 48 genes have been associated with autosomal dominant nonsydnromic hearing loss mentioned in hereditary hearing loss as (hereditaryhearingloss.org) as summarized in **Annex Table 2**.

There are 76 genes implicated in autosomal recessive non-syndromic hearing loss (ARNSHL) summarized in **Annex Table 3**. The most common cause of severe-to-profound ARNSHL in most populations is the mutation of *GJB2* gene, recent studies have shown that the contribution of pathogenic variants in gap junction protein beta 2 (*GJB2*) to deafness varies considerably by ethnicity [42]. For example, among individuals of African descent, pathogenic variants in *GJB2* are very rare [45]. The most common cause of mild-to-moderate autosomal

recessive hearing loss is a mutation of the S*TRC* gene [42]. In general, the hearing loss associated with dominant inheritance is less severe than that associated with recessive inheritance [44].

The X-linked, Y-linked and mitochondrial are rare cases for causing HL [40,41,46]. In X-linked NSHL there are only three genes found to be associated with it; PRPS1 (MIM 311850; DFNX1, formerly DFN2) [47], POU3F4 (MIM 300039; DFNX2, formerly DFN3) [48] and small muscle protein X-linked (SMPX) [49]. The Y-linked NSHL (DFNY1, 400043) was reported only once in a Chinese family [50]. Finally, the mitochondrial NSHL is mainly caused by maternal inheritance. MT-RNR1 and MT-TS1 are the genes contributed with the mitochondrial NSHL [51].

1.3 Genes involved in hearing loss

There are approximately 40 genes reported as the cause of NSHL in the middle east [52]. The genes involved can be divided to: genes involved in cochlear homeostasis such in gap and tight junctions, genes implicated in cellular organization, genes coding for tectorial membrane-associated proteins, genes involved in neuronal transmission, genes implicated in cell growth, differentiation and survival, and genes with others or unknown functions [52]. The following are the genes identified and have been involved in our research.

1.3.1 Genes involved in cochlear homeostasis

1.3.1.1 Gap junctions

There are three genes listed under the gap junction: gap junction protein beta 2 gene (*GJB2*) which encodes the gap junction channel protein connexin 26 (Cx26), gap junction protein beta 6 gene (*GJB6*) which encodes the gap junction channel protein connexin 30 (Cx30) and gap junction protein beta 3 gene (*GJB3*) which encodes the gap junction channel protein connexin 31 (Cx31).

Gap junctions are clusters of intercellular channels that allow for direct communication between cells [53,54]. Cx26 is a member of the family related gap-chain-forming proteins, each of which is commonly referred to as molecular weight (Cx26, Cx30, etc.). Genes for 20 different proteins are present in the human genome [54].

The mutation in the GJB2 is the most common cause of hearing loss [13] and it is the most prevalent for congenital HL [41,55]. The mutation spectrum also varies among different ethnic groups [56]. Researchers have shown that mutations in the encoded protein, Connexin 26 (Cx26), account for half of the recessive deafness in many different populations [55,57,58] and, a previously reported study on 22 Arab countries, revealed that most of the reported variants occurred is in the GJB2 with 21 different variants (20%) [59].

One specific mutation, c.35delG, a truncating mutation, accounted for the majority of GJB2 mutations observed in Mediterranean populations and it is the most prevalent variant associated with non-syndromic autosomal-recessive hearing loss [60]. The deletion mutation (c.35delG) is a frameshift mutation that terminates the Connexin 26 protein polypeptide chain prematurely (p. Gly12Valfs*2). In the three GCC nations, Qatar, Oman, and KSA, c.35delG was equal to 3.2 %, 0 %, and 6.7 % respectively [61–63]. In the U.A.E, mutations in the GJB2 gene were associated with deafness in nine patients (18%). Eight deaf people had a mutation of c.35delG, of which six were homozygous and three were heterozygous [64].

Throughout the Middle East, the c.35delG mutation predominates throughout Turkey, northern Iraq, and northwest Iran; however, it is less prevalent in Pakistan, India, Qatar, Oman, and KSA [65]. As the mutation within the Cx26 and specifically the deletion (c.35delG) is the most common cause of NSHL in the middle east and in the U.A.E, we first screen all the samples using CX26 primers when looking for novel genes related to HL and only samples with no GJB2 gene is selected for further analysis.

1.3.1.2 TIGHT JUNCTIONS

Beyond the gap junctions, the tight junctions in the inner ear are also important; they are essential to sustain the paracellular permeability between endolymph and the adjacent tissue and maintain the apical-basal polarity within the cell [66]. Thus, tight junctions are considered a dynamic barrier between the external and internal environments.

Claudins are integral membrane proteins of all epithelia and endothelia found in tight junctions. Claudins were first reported by Furuse in the late Shoichiro Tsukita laboratory in 1998 in a diluted junctional fraction from the chicken liver [67]. They seem to be the close junction's essential structural components. The over-expression of claudins in fibroblasts, which usually lack

near junctions, is necessary to reconstitute tight junction-like strand networks [68]. It is now also clear that claudins are both paracellular barriers and pores, and thus play a key role in determining the properties of epithelial and endothelial cells' permeability.

The key support for this comes from a multitude of studies that indicate the overexpression, knockdown, or knock-out of claudins and both naturally occurring and experimentally induced claudin mutations, or those introduce cysteines which are accessible by extra-cellular probes for covalent modification, indicate that the extracellular domains of claudins must line the paracellular pore [69].

Claudin, which consists of 24 members, has a specific expression of tissue and is highly expressed in the kidney, liver, and inner ear [70,71]. Claudins variety is approximately from 22–24 kDa with 4 transmembrane domains. They have giant protein sequence similarity to claudins-1 and -2, and carboxyl termini diagnosed with the aid of PDZ domains of the sub- membranous plaque proteins ZO-1, ZO -2, ZO -3 [55]. Hydropathy plots indicate four transmembrane helices (TM1–4) and the general structure of all claudins consists of an intracellular NH2 terminus which is very short, with the possible exception of claudin-5, -16 and -25, together with a longer intracellular COOH terminus, two extracellular loops (ECL1, which is larger and a smaller ECL2) and one short intracellular loop terminus. The differences lie exclusively within the intracellular NH2 termini of these proteins (claudin-5, 7 vs. 92 amino acids; claudin-16, 3 vs. 73 amino acids; claudin-25, 4 vs. 27 amino acids), and the version of which is the physiologically relevant form that remains a matter of debate.

Other typical features of the claudin family include a sequence of the signature within ECL1, and a COOH-terminal PDZ-binding motif, through which the majority of human claudins [56] with the probable exception of claudin-12, claudin-19a, claudin-21, and claudin -24 to claudin -27 [57] , may interact with PDZ domains associated with the tight-junction scaffolding / adapter proteins. PDZ stands for PSD95 (post-synaptic protein density), Dlg1 (drosophila disk wide tumor suppressor), and ZO-1 (zonula occludens-1 receptor), the first three proteins to be identified in such domains. Tight PDZ domain proteins associated with the junction include ZO-1, ZO -2, and ZO -3, MUPP1, and MAGI-1 to MAGI-3. These adapter proteins also bind actin directly or indirectly, thus anchoring the close junction inside the cytoskeleton [58].

The mammalian claudin group of twenty-seven qualities encodes tight intersection proteins that capacity to keep up the trustworthiness of the apical and basolateral layer areas and forestall

dispersion of solutes and dissolvable particles through intercellular spaces inside epithelial sheets [59–62]. The claudin proteins are anticipated to have four transmembrane areas and short cytosolic amino and carboxy termini [63,64]. While the first and the fourth transmembrane districts just as the extracellular circles are exceptionally rationed among the distinctive claudin species, the second and the third transmembrane locales are variable [65].The main extracellular circle of these proteins has a significant job in homophilic connections **(Figure 2)** [66] and as shown in the figure 2, the greater part of the known transformations of claudin 14 (CLDN14) ,is inside or near the second or third transmembrane spaces and some of them have been appeared to influence transmembrane localization.

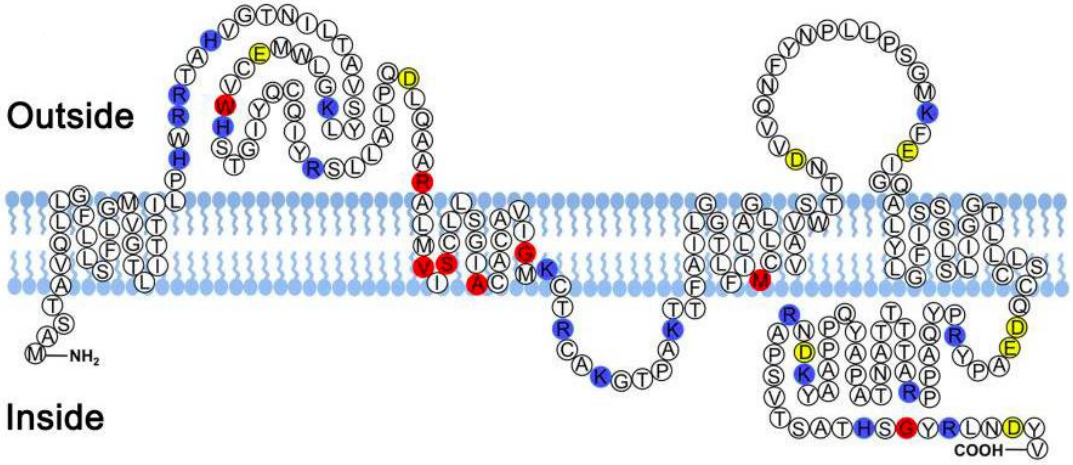

FIGURE 2. SCHEMATIC OF HUMAN CLAUDIN14. Topology of CLDN14 was anticipated by Tmpred code. Yellow and blue are amino acids indicating negatively and positively charged residues. Figure from [67]

Claudin mutations are known to cause four Mendelian inherited disorders, neonatal sclerosing cholangitis with ichthyosis (claudin 1 mutations, OMIM 607626), autosomal recessive, non-syndromic deafness (claudin-14, OMIM 614035), family hypomagnesemia hyper calcinosis (FHHNC) with nephrocalcinosis (claudin-16 mutations, OMIM 248250), and ocular involve FHHNC (claudin-19, OMIM 2). Additionally, polymorphisms were found to be associated with polygenic diseases in claudin genes, including claudine-1 with atopic dermatitis [68] claudine-5 with schizophrenia [69,70] and claudine-14 (*CLDN14*) in kidney stone disease [71].

Also, several different genes including tight junctions *TRIC* and *CLDN14* are involved in inner ear ion homeostasis and have been linked to deafness. To be precise, Changes of *CLDN14* cause autosomal passive nonsyndromic deafness at the DFNB29 locus. Until this point in time, six

diverse pathogenic variations of human *CLDN14* have been distinguished in families with isolating serious to significant hearing misfortune, yet no undeniable vestibular phenotype [72–75]. Similarly, a *CLDN14* knockout mouse is additionally deaf [76]. Although claudin 14 is communicated in the mouse vestibular tactile epithelium, *CLDN14* knockout mice seem to have no conspicuous vestibular issue, for example, revolving around conduct or head-bobbing [76].

The *CLDN14* (NM 012130) gene consists of three exons, encoding 239 amino acid residues for a protein [72].In the inner ear, *CLDN14* plays a key role during the auditory process by recycling potassium ions from the hair cells back to endolymph [77]. It has been hypothesized that the absence of Claudin 14 from tight junctions in the organ of Corti leads to altered ionic permeability of the paracellular barrier of the reticular lamina and that prolonged exposure of the basolateral membranes of outer hair cells to excessive potassium concentrations can be the motive of the demise of hair cells which can be related to hearing loss [78]. In addition as reported tight junctions have been shown to play a significant role in preserving the structural integrity of the cells in the inner ear [79]. The structure of the most intercellular tight junctions in the internal ear is comparable to that reported in different epithelia [80–83]. However, the structure of the bicellular junctions between hair cell and supporting cells, particularly between an outer hair cell and adjoining Deiter's cell is extra complex and extraordinarily specialized to hold the ionic barrier between endolymph and perilymph [81–83]. These tight junctions include a high amount of claudin 14 and are prominently stained with anti-claudin 14 antibodies **(Figure 3 A-B).** The apical junctional complexes between the cells of the organ of Corti lack desmosomes and hole junctions and have an aggregate of tight junction and adherens junction points and extend down the depth of the reticular lamina, an area spanning 3-5 μm [83].

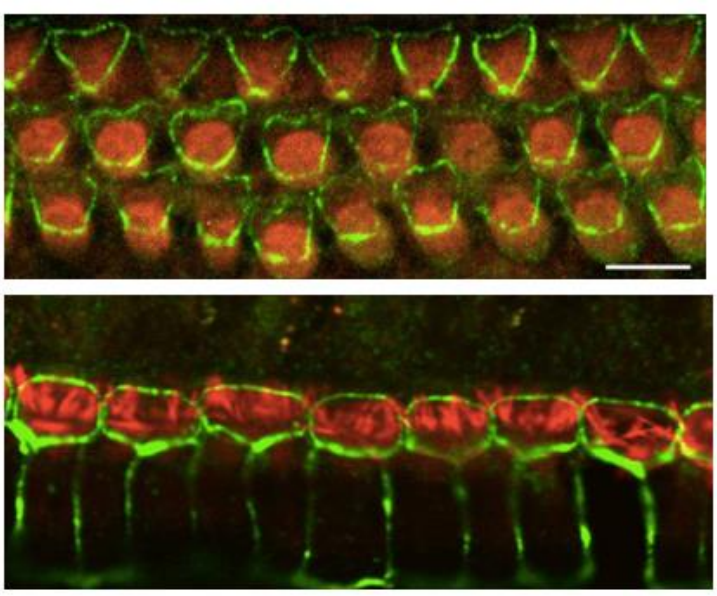

FIGURE 3. CLDN14 LOCALIZATION IN OUTHER AND DEITHERS'S CELLS. (A) **Localization of CLDN14 in the apical bicellular tight junctions between the outer cells (ohcs) and deithers's cells (green).** (B) **localization of CLDN14 (green) in the tight junctions between inner hair cells (ihcs) and pillar cells, and between two adjacent pillar cells. filamentous actin is highlighted by rhodamine-phalloidin (red). figure from [67].**

1.3.2 GENES WITH OTHER OR UNKNOWN FUNCTIONS

1.3.2.1 MITOCHONDRIAL DNA

The mitochondrial genome is the complete genetic contribution of a mitochondria. Mitochondrial DNA is just a small part of a eukaryotic cell 's overall DNA and is inherited exclusively from a mother.

Mitochondria is an independent organelle containing its own genomic DNA separated from nuclear DNA and found to be floating in the cytoplasm; referred to as the cell's powerhouse, since it supplies the cell with energy in the form of adenosine triphosphate (ATP) from oxygen during respiration in order to perform its specific functions efficiently. Its genome is 16569 bp in size encoding 37 genes, has several hundred copies per cell, and consists of heavy and light strand (based on the proportion of higher molecular weight nucleotides). Heavy strand contains 2 rRNA sequences, 14 of the 22 tRNA, d-loop and polypeptides, all of which have no introns and all protein

encoding genes with the exception of ND6 **(Figure 4)** [84]. There is also a wide non-coding area of about 1 kb, which includes regulatory elements for the initiation and termination of transcription of both strands. The D-Loop (displacement loop) or control area is within this non-coding region and contains the OriH (heavy-strand replication origin) where replication is within it. There is a second source of duplication in the line of light, the OriL **(Figure 4)** [85].

The mitochondria are two-membrane-bound spherical organelles which rely on the different functions of two membranes: the external mitochondrial membrane and the internal mitochondrial membrane. A large multiprotein translocases network that recognizes mitochondrial signal sequences of larger proteins and allows their movement. The outer mitochondrial diaphragm includes porins that permit smaller than 5 kilodalton molecules to travel. The inner mitochondrial membrane includes all the components of the electron transport system and the ATP complex; the mitochondrial membrane also has several invaginations, known as cristae, which considerably increase its total size. The double membranes form two mitochondrial compartments: the inner and outer membrane space of the intermembrane and the inner membrane of the matrix. Within the mitochondrial matrix, mitochondrial DNA is located [86].

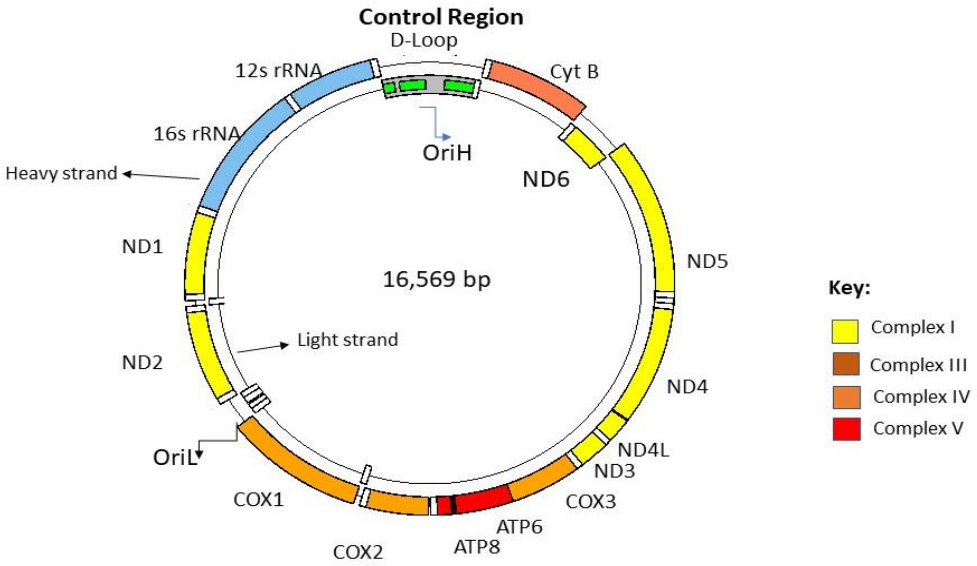

FIGURE 4. SIMPLIFIED FIGURE OF HUMAN MITOCHONDRIAL GENOME. Adapted from [87]

Mitochondrial genetics is distinct from Mendelian genetic in nearly every aspect of it *(***Table 1)** [88]. Owing to this maternal descent, the origin of the disease can only be transmitted along the matrilineal line. Since mitochondria have a low-activity DNA repair mechanism and no histones and are continuously exposed to oxygen radicals leaked from the mitochondrial electron transfer chain, somatic mutations in mitochondrial DNA (mtDNA) are normal. Typically, most healthy individuals tend to have only one mtDNA genotype (i.e., are homoplasmic) but, in many mitochondrial disease states, there are mixed mtDNA genotypes(i.e. heteroplastic) [88]. The amount of heteroplasmy varies depending from tissue to tissue, and from cells within the tissue. The extent of the symptoms is not always well linked to the share of mutant mitochondrial chromosomes since different species rely in a different way on mitochondrial OXPHOS. The altered clinical phenotypes may be the result of organ-specific energy impairment [89]. Phenotype heterogeneity also exists in the case of homoplasmy, which is likely to result from different nuclear genetic backgrounds or mitochondrial haplotypes. Comprehensive reviews of normal mitochondrial genetics were published [88].

TABLE 1. COMPARISON BETWEEN HUMAN NUCLEAR GENOMES AND MITOCHONDRIAL GENOMES*

Characteristic	Nuclear genome	Mitochondrial genome
Size	~3 x 10^9 bp	16,569 bp
Shape	Linear double helix	Circular double helix
Inheritance	Both parents	Maternal
Mode of inheritance	Mendelian inheritance for autosomes and the X chromosome; paternal inheritance for the Y chromosome	Exclusively maternal
Number of DNA molecules per cell	23 in haploid cells; 46 in diploid cells	Several thousand copies per cell (polyploidy)
Number of genes	~20,000 protein coding	37 (13 polypeptides, 22 tRNAs and 2 rRNAs)
Gene density	~1 per 40,000 bp	1 per 450 bp
Introns	Frequently found in most genes	Absent
Histones	Associated with the DNA	Not associated with the DNA
Associated proteins	Nucleosome-associated histone proteins and non-histone proteins	No histones; but associated with several proteins (for example, TFAM) that form nucleoids
Percentage of coding DNA	~3%	~93%
Codon usage	The universal genetic code	AUA codes for methionine; TGA codes for tryptophan; AGA and AGG specify stop codons
Replication	Strand-coupled mechanism that uses DNA polymerases α and δ	Strand-coupled and strand-displacement models; only uses DNA polymerase γ
Transcription	Most genes are transcribed individually	All genes on both strands are transcribed as large polycistrons
Recombination	Each pair of homologues recombines during the prophase of meiosis	There is evidence that recombination occurs at a cellular level but little evidence that it occurs at a population level
Methylation	Present (3-4% of all Cs (~ 70-80% of all CpGs)) (mainly CpG)	present (~ 1.5-5% of all Cs) (both CpG and CnonG)

*Table modified from [88] and [90]

Mitochondrial disorders can be inherited or acquired. Inherited mtDNA mutations are responsible for several clinically defects, including multiple types of neuropathy, myopathy, cardiomyopathy, retinal degeneration, diabetes mellitus, and sensorineural hearing loss (SNHL) [12,91–94]. Progressive hearing loss may be one of the symptoms in many patients with classical mitochondrial disorders, such as myoclonic epilepsy and ragged red fiber (MERRF) [95], Kearns-Sayre Syndrome (KSS) [96] , and mitochondrial encephalopathy with lactic acidosis and stroke-like episodes (MELAS) [97]. The only symptom that mitochondrial dysfunction can be found is hearing loss. This shows that hearing relies heavily on mitochondrial function [101]. Inherited deafness-associated mtDNA mutations typically occur in protein encoding genes or protein synthesizing apparatus components, rRNAs and tRNAs.

MtDNA mutations are one of the most significant causes of sensorineural hearing loss, especially in the 12S ribosomal RNA (*12S rRNA*) gene. Mitochondrial encoded 12S ribosomal RNA (often abbreviated as 12S or 12S rRNA), also known as mitochondrial-derived peptide MOTS-c or mitochondrial open reading frame 12S rRNA-c is a component of the small mitochondrial ribosome subunit (SSU). In humans, 12S is encoded with the MT-RNR1 gene and is 959 nucleotides long [98,99].SNHL-associated mitochondrial rRNA mutations were found only in the *12S rRNA* gene. The mutation origins in this gene include A1555 G [100–103], C1494 T [104], T1095C [105–108], A827 G [109,110] and 961 mutations [105,106,111–114]. Aminoglycosides and SNHL can cause mutation in the *12S rRNA* gene [115–117]. No surface-associated mutations are found in the mitochondrial *16S rRNA* gene.

1.3.2.2 TRANSMEMBRANE CHANNEL LIKE 1

Transmembrane channel-like protein 1 is a protein that is encoded by the TMC1 gene in humans [115–117]. TMC1 comprises six transmembrane domains with both the C and N terminals on the endoplasmic side of the membrane, as well as a wide loop between domains 4 and 5. This topology is close to that of transient receptor potential channels (TRPs) [115], a family of proteins involved in sensory sensations such as temperature, taste, sound, and vision [118]. TMC1 has been found in postnatal mouse cochlea and TMC1 and TMC2 knockouts have resulted in both auditory and vestibular defects (hearing loss and balance issues) suggesting that TMC1 is a molecular part of auditory transduction [119].

Mutations of the *TMC1* gene have been shown to cause autosomal dominant and recessive NSHL forms associated with the DFNA36 and DFNB7 / B11 loci, respectively. TMC1 has previously been associated with both progressive and permanent hearing impairment [118]. It was proposed that TMC1 may be an ion channel or transporter that mediates K+ homeostasis in the inner ear. TMC1 was initially mapped to chromosome 9q13–q21 in two consanguineous Indian families with a pre-lingual, severe-to - profound description of the DFNB7 locus [119].

Different studies suggested that the *TMC1* gene is one of the five major genes causing profound recessive deafness worldwide [118,120–122]. Mutations in this gene account 3% to 6% of deafness in Turkish Tunisian, European, Indian and Pakistani populations [123,124].

2.RESULTS

2.1 ARTICLE 1

A Novel Nonsense Mutation (c.414G>A; p.Trp138*) in *CLDN14* Causes Hearing Loss in Yemeni Families: A Case Report

Walaa Kamal Eldin Mohamed, Mona Mahfood, Abdullah Almutery, Sallam A.Hassan, Abdelaziz Tlili
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Deafness is the most predominant hereditary sensorineural disease worldwide. In the Arab population due to the consanguineous marriages, the rate of hereditary diseases like nonsyndromic hearing loss (NSHL) is high. Scientists so far identified more than 90 genes associated with NSHL. Some of these genes are involved in the development and the function of the inner ear. Some genes are involved in the cochlear homeostasis; like the gap and tight junctions. Gap junction protein beta 2 (*GJB2*) gene is the most common gene responsible for NSHL. Claudin-14 (*CLDN14*) gene which is listed under tight junction is an effective tight junction molecule for a wide range of epithelial physiologies. CLDN14 mutations have been found in both uncommon (monogenic) and common (polygenic) types of human genetic diseases. Although preliminary studies have suggested the function of CLDN14 as a cation blocker in the inner ear.

All the affected individuals were screened for GJB2 mutation and only individuals with no GJB2 mutation were selected for further analysis. The Clinical exome sequencing helped to identify the novel nonsense mutation in the CLDN14 gene. Bioinformatics analysis and molecular techniques are used to confirm the nonsense mutation. Also, as this was the first study to screen for a mutation causing hearing loss in Yemen, the microsatellite markers used to reveal the founder effect of the nonsense mutation.

In conclusion, the study indicates the presence and the role of the *CLDN14* gene in the Yemeni population as causative of NSHL.

frontiers in Genetics

CASE REPORT
published: 08 November 2019
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A Novel Nonsense Mutation (c.414G>A; p.Trp138*) in *CLDN14* Causes Hearing Loss in Yemeni Families: A Case Report

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Non-syndromic hearing loss (NSHL) is a hereditary disorder that affects many populations. Many genes are involved in NSHL and the mutational load of these genes often differs among ethnic groups. Claudin-14 (*CLDN14*), a tight junction protein, is known to be associated with NSHL in many populations. In this study, we aimed to identify the responsible variants in 3 different Yemeni families affected with NSHL. Firstly, clinical exome sequencing (CES) performed for 3 affected patients from these different families identified a new nonsense variant (c.414G > A) in *CLDN14*. This variant was then confirmed by Sanger sequencing and PCR-RFLP. Subsequently, four microsatellite markers were used to genotype these families, which revealed a founder effect for this variant. Overall, this study illustrates the implication of the *CLDN14* gene in the Yemeni population with NSHL and identifies a new founder variant.

Keywords: *CLDN14* gene, clinical exome sequencing, nonsense variant, non-syndromic hearing loss, founder effect

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BACKGROUND

Hearing loss (HL) is a relatively common congenital disorder; its prevalence in newborns is 1 in 1,000 live births (MORTON, 1991). Hereditary HL can be associated with other symptoms but in the majority of cases (70%) it is non-syndromic (Najmabadi and Kahrizi, 2014). Non-syndromic hearing loss (NSHL) can be classified by its mode of inheritance as; autosomal dominant (~15–20%), autosomal recessive (~80%), X-linked, Y-linked or mitochondrial (together ~2%) (ACMG; Hone and Smith, 2002).

Autosomal recessive non-syndromic hearing loss (ARNSHL) is extremely genetically heterogeneous with 72 genes and 108 loci associated with this type of HL (https://hereditaryhearingloss.org). Next Generation Sequencing (NGS) is an important approach for screening diseases with high heterogeneity, such as hearing loss, as it can quickly characterize hundreds of genes and a large number of common variants (http://hearing.harvard.edu/db/genelist.htm) in timely and cost effective manner as opposed to Sanger sequencing (Gürtler et al., 2008; Gao et al., 2015).

Abbreviations: NSHL, Non-syndromic Hearing Loss; ARNSHL, Autosomal Recessive Non-Syndromic Hearing Loss; HL, Hearing Loss; PCR, Polymerase Chain Reaction; RFLP, Restriction Fragment Length Polymorphism; *CLDN14*, Claudin 14; CES, Clinical exome sequencing.

Variants in gap and tight junctions are known to be associated with ARNSHL (Martínez et al., 2009; Kim et al., 2014). Connexins are important for the formation of gap junctions between adjacent cells, where direct intercellular communication *via* diffusion of ions and metabolites can occur (Goodenough and Paul, 2003); members of the connexin family have an important role in ARNSHL. In fact, many reports claim that approximately 50% of autosomal recessive HL cases are caused by either homozygous or compound heterozygous variants in the gap junction beta 2 (*GJB2*) gene (Zheng et al., 2015), making the *GJB2* gene the most frequent gene implicated in ARNSHL. Therefore, it is common to pre-screen NSHL individuals for *GJB2* variants, to determine whether NGS techniques are required. In the inner ear, tight junctions are involved to maintain the difference in concentration between the endolymph and the perilymph. The *CLDN14* gene, encoding a tight junction protein, serves as potassium-restrictive barrier in the Corti organ (Ben-Yosef et al., 2003). Since the identification of *CLDN14* variants in families with ARNSHL (Wilcox et al., 2001), several genetic and functional studies have been performed. These studies confirmed the implication of the *CLDN14* gene in hearing loss (Lu et al., 2018).

In this study, we performed Clinical Exome Sequencing (CES) and identified a new nonsense variant in three different Yemeni families. Haplotype analysis of the *CLDN14* gene region, using four microsatellite markers, showed that this pathogenic DNA variant has a founder effect.

CASE PRESENTATION

In this study, we investigated three consanguineous Yemeni families diagnosed with ARNSHL (**Figure 1A**). The proband YMN2 of the family YMN-I is a 25 year old affected female. Her audiogram showed an average pure tone air conduction (PTA)

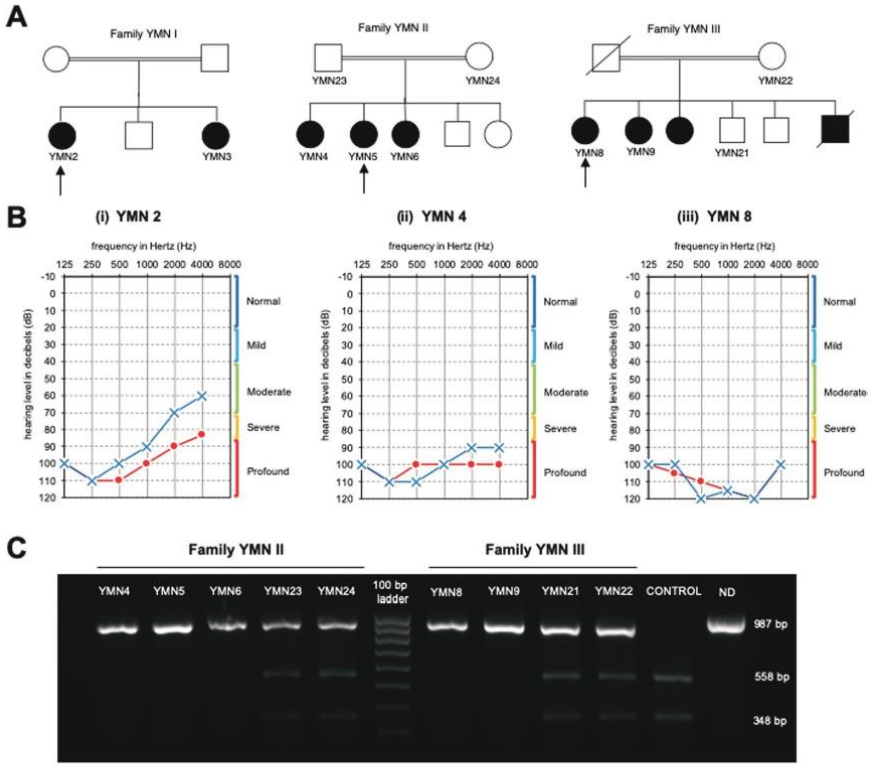

FIGURE 1 | Pedigrees of the affected families, Audiograms and PCR-RFLP. **(A)** Pedigrees of three affected families with non-syndromic hearing loss. Arrows denote the probands. **(B)** Audiograms of the affected probands from the 3 Yemeni families: (o) indicates air conduction for right ear, (x) indicates air conduction for left ear. **(C)** Results of the PCR-RFLP analysis in YMNII and YMNIII affected families. The 987 PCR product digested with the AvaII restriction enzyme. The wildtype allele is cleaved into three fragments 558, 348, and 81 bp, whereas the c.414G>A mutant allele is cleaved into two fragments 906 and 81 bp in length. ND, undigested PCR product.

varying from 60–80 for her left and right ears (**Figure 1**) and the otoscopy of both ears was normal. The proband's younger sister (YMN3) is 23 years old and showed an average PTA of 70–80; her otoscopy was also normal (**Supplementary Figure 1**). The proband's younger brother and parents are phenotypically normal. The YMN-II family consists of three affected female siblings, YMN4 is 29 year old, YMN5 is 14 year old and YMN6 is 21 year old with PTA of 90–100, 70–80 and 80 for left and right ears respectively, their otoscopy was also normal. Moreover, the proband's third sister, brother and parents are phenotypically normal (**Figure 1** and **Supplementary Figure 1**). The last family YMN-III consisted of two affected female siblings YMN8 and YMN9 (25 and 29 years old respectively) with PTA of 100, 90–100 for left and right ears (**Figure 1** and **Supplementary Figure 1**), the otoscopy result for both affected females was normal. The proband in this family has an elder affected sister (sample could not be collected), deceased affected brother, two phenotypically normal brothers and parents. In summary, the analysis of the audiograms of the three families (**Supplementary Table 1**), showed a sloppy audiogram shape in all the affected individuals.

MATERIALS AND METHODS

Sample Collection

Collection of samples was done in collaboration with Al-Amal association of deaf and mutism in Hadramout coast (Mukallah, Yemen). Consent forms were filled and signed by all participants. Saliva samples were collected from the participants using Oragene-DNA (OG-500) Kit (DNA Genotek, CANADA), and only letters and numbers were used to label DNA samples to protect the participant's privacy. Genomic DNA was extracted using Prep IT L2P (DNA Genotek, CANADA) following the manufacturer's protocol. The experimental procedures were approved by the Ethics Committee from the University of Sharjah (Sharjah, UAE) and by the General Directorate of the Office of the Ministry of Health and population-Hadramout Coast (Mukallah, Yemen).

Clinical Exome Sequencing and Bioinformatics Analysis

Clinical exome sequencing and standard data analysis was performed for the affected individuals YMN3, YMN5, and YMN8. The genomic DNA was sheared and used to perform exome capture using oligonucleotide probes (SureSelect V5+UTRs) following the manufacturer's provided protocols. The exonic region was later enriched by hybridizing capture probes. The captured fragments were then adapted to produce libraries that were sequenced on the Illumina HiSeq 2500/4000 system (Illumina, San Diego, CA, USA) to generate paired end 2X 100bp sequence reads to produce 100x mean coverage. Only reads that were generated from high quality sequences were analyzed after quality control for variant calling and annotation, and later were aligned to the human reference genome build GRCh37/hg19 using BWA-0.7.12. PCR duplicates were then removed using Picard-1.140 and variants were called using Genome Analysis Toolkit (GATK) v2.3-9. Known variants were annotated with in-house Variation and Mutation Annotation Toolkit 2.3.4 (VariMAT). The remaining variants were then further filtered by frequency (i.e. < 0.01%) in dbSNP (https://www.ncbi.nlm.nih.gov/projects/SNP/) or ExAC Browser (http://exac.broadinstitute.org/). Finally, to predict the functional impact of the candidate variants the following bioinformatics tools were used: Variant Effect Predictor (VEP) (http://grch37.ensembl.org/Homo_sapiens/Tools/VEP) and VarSome (https://varsome.com/).

Sanger Sequencing

Sanger sequencing was performed for all members of the investigated families to confirm the co-segregation of the c.414G>A variant with the disease phenotype. In order to amplify the exonic region corresponding to this variant, we designed the following primers: CLDN14-F: ACCACCATCCTGCCGCACTG and CLDN14-R: TGTTTGCAGTGGTCGTGGTG. Polymerase Chain Reaction (PCR) was then carried out at an annealing temperature of 55°C generating amplicons of 550 bp in length. The amplified products were then purified using ExoSapIT clean up reagent (Affymetrix, Fisher Scientific, Göteborg—Sweden). Cycle sequencing was performed using Big dye terminator V3.1 cycle sequencing kit (Applied Biosystems, USA). The products were later purified using Dye EX 2.0 spin kit (Qiagen, Germany) and injected in the 3500 Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, USA). The sequences were aligned with the published sequence of the *CLDN14* gene (NG_011777.1; NP_001139549.1).

Variant Screening Using PCR-RFLP

The AvaII restriction enzyme was used to screen the c.414G>A nucleotide transition in the affected families and healthy controls. The primers' sequences were: CATTTCCTTTCTCTCCCTGCT and GACATTTCCTCGCATTCACA. The PCR product size was of 987 bp. The digestion of PCR products was performed according to the manufacturer's instructions (New England Biolabs, USA) followed by separation on 2% agarose gel at 80 V for 1 h and half.

Genotyping

Four microsatellite markers (D21S1252, D21S168, D21S267, and D21S1894), flanking the *CLDN14* gene chromosomal region were chosen (**Figure 3A**). The sequences of these microsatellite markers are described in **Supplementary Table 2**. For each marker, the forward primer was labeled with a specific fluorescence and the PCR products were analyzed using the Genetic Analyzer 3500 (Applied Biosystems, Thermo Fisher Scientific, USA). Gene mapper software v5.0 was used for alleles call and haplotypes were constructed manually.

RESULTS

Clinical Exome Sequencing (CES) Analysis

Three affected families with ARNSHL were studied (**Figure 1A**). The proband of each affected family was screened for variants in the *GJB2* gene using PCR and Sanger Sequencing and no variant has been detected. Thus, we performed a CES analysis including

126 genes implicated in HL. The total numbers of variants were 327640 for YMN3, 314150 for YMN5, and 267190 for YMN8. As all families were consanguineous, we considered only homozygous variants located in the HL-related genes and with a frequency less than 0.01 (**Table 1**). Subsequently, VEP (Variant Effect Predictor) tool, LoFTool score, LRT pred, Mutation Taster pred and VarSome were used to predict the functional impact of these remaining variants. The LoFTool score identified only one variant in the *CLDN14* gene (c.414G>A) as damaging, LRT pred showed that the same variant is D (deleterious) and the Mutation Taster pred showed that it is a disease causing (D, D, D, D). VarSome also confirmed that this variant in the *CLDN14* gene is predicted to be pathogenic.

To confirm these findings, we sequenced the 7th exon of the *CLDN14* using Sanger sequencing (**Figure 2**). The analysis revealed a co-segregation of the c.414G>A variant in the three families. This co-segregation was also confirmed by PCR-RFLP using the AvaII restriction enzyme (**Figure 1C**). In fact, all affected individuals were homozygous for this nucleotide transition, parents were heterozygous, and unaffected siblings were either heterozygous or homozygous normal.

Genotyping and Haplotype Analysis

Based on the previous results shown in **Table 1**, we assumed that the nonsense variant p.Trp138Ter (c.414G>A) is a common founder variant. To confirm our hypothesis, four short tandem repeats (STR) markers (D21S1252, D21S168, D21S267, D21S1894) were analyzed in the three affected families (**Figure 3A**). Apart from marker D21S1894, a homozygous haplotype for the alleles 246,104 and 188, of the markers D21S1252, D21S168, and D21S267, has been detected in all affected individuals, while parents and healthy individuals were heterozygous for this particular haplotype (**Figure 3B**).

DISCUSSION

In this study, we analyzed 3 Yemeni families with ARNSHL. As variants in the *GJB2* gene are among the most common variants in Middle East (Shahin et al., 2002; Tlili et al., 2017), known to be related to HL and is commonly used for clinical screening (Zheng et al., 2015), we first pre-screened the affected individuals for variants in this gene. Due to the absence of pathogenic variants within the *GJB2* gene, we further analyzed the affected samples by CES. The CES results allowed us to identify a novel nonsense variant p.Trp138Ter (c.414G>A) in the *CLDN14* gene. The co-segregation of this variant with the disease was confirmed by Sanger sequencing and PCR-RFLP. While most of the variants in *CLDN14* that lead to hearing loss in different populations were missense variants (**Supplementary Table 3**), nonsense variants were only found in this study and in one family from Pakistan (Lee et al., 2012). Variants of *CLDN14* have been previously associated with hearing loss in Canada, Pakistan, India and Morocco (Wilcox et al., 2001; Bashir et al., 2010; Lee et al., 2012; Bashir et al., 2013; Charif et al., 2013; Pandey et al., 2017; Pater et al., 2017) (**Supplementary Table 3**). In addition, some studies showed the absence of variants in the *CLDN14* gene for other

TABLE 1 | Predicted impact of the remaining variants after filtration of the CES results.

Gene	Patient ID	Variant type	cDNA variant	Amino acid change	Classification
BDP1	YMN3	Exonic-NC*	n.2439A>T	*NA	Likely benign
CLDN14	YMN3	Nonsense	c.414G>A	p.Trp138Ter	Pathogenic
	YMN5				
	YMN8				
SLC26A2	YMN5	Missense	c.1721T>C	p.Ile574Thr	Benign
NARS2	YMN5	5UTR	c.-854C>T	*NA	Uncertain significance
CDH23	YMN8	5UTR	c.-35_-31dup	*NA	Likely benign

*NC, Exonic Non coding; *NA, Not available.*

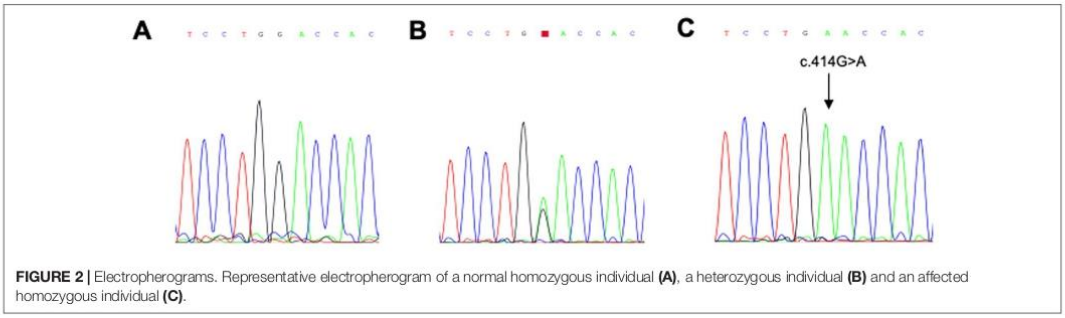

FIGURE 2 | Electropherograms. Representative electropherogram of a normal homozygous individual **(A)**, a heterozygous individual **(B)** and an affected homozygous individual **(C)**.

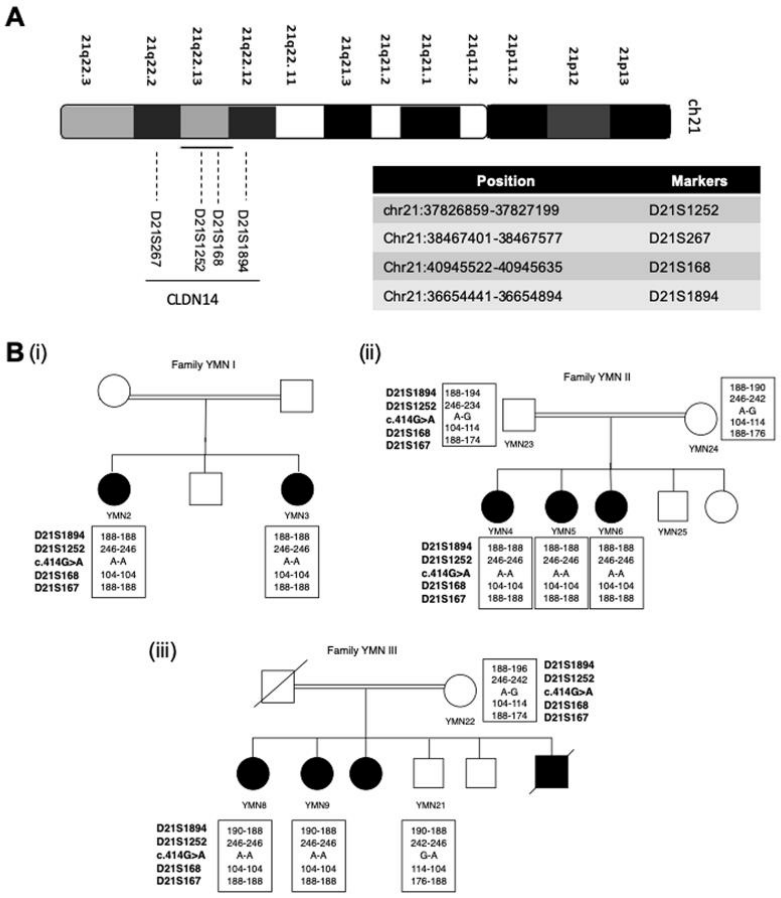

FIGURE 3 | Founder effect of the c.414G>A variant. **(A)** Chromosomal location of the *CLDN14* gene and the 4 STR markers on chromosome 21 used to study the c.414G > A founder effect **(B)** Haplotype analysis in the 3 affected families showing the segregation of a common haplotype with the c.414G>A variant and confirming its founder effect.

populations such as Tunisia, Turkey and China (Uyguner et al., 2003; Belguith et al., 2009; Lu et al., 2018).

The new c.414G>A variant within the *CLDN14* gene showed a phenotypic variability among the 3 Yemeni families. In fact, the audiograms revealed that the affected members have moderate to profound congenital hearing loss (**Figure 1B**). The HL phenotypic variability observed in our study has been also reported in different families with *CLDN14* variants (Wilcox et al., 2001). A previous study that compared the audiograms of affected Pakistani individuals with *CLDN14* variants, showed that the studied patients presented moderate to severe, moderately severe to profound, and severe to profound HL. This phenotypic variability could be explained by both the nature of the pathogenic variant and the possibility of interaction with modifier genes (Bashir et al., 2010; Bashir et al., 2013). For the Yemeni families studied here, and as the variant is common, the phenotypic variability is probably due to modifier genes and/or environmental factors.

Tight junctions in the inner ear are important as they are essential to sustain the paracellular permeability between endolymph and the adjacent tissue and maintain the apical-basal polarity within the cell. Thus, tight junctions are considered a dynamic barrier between the external and internal environments (Gupta and Ryan, 2010). Variants occurring in tight junction genes such as claudin-14 (*CLDN14*) have been shown to be involved in HL (Nunes et al., 2012). Claudin 14 is highly expressed in the kidney, liver and inner ear (Wilcox et al., 2001; Ben-Yosef et al., 2003). In the inner ear, *CLDN14* plays a key role during the auditory process by recycling potassium ions from the hair cells back to the endolymph (Duman

and Tekin, 2012). *In vitro* studies, mouse knockout models and human phenotype studies demonstrated that this gene is very crucial for a normal function of the auditory system (Wilcox et al., 2001; Ben-Yosef et al., 2003; Angelow et al., 2008). In fact, it has been shown that *Cldn14*-null mice exhibit HL due to the rapid deterioration and the loss of function of their cochlear outer hair cells (OHCs) shortly after birth (Ben-Yosef et al., 2003).

Since the c.414G>A variant was observed in three unrelated families, we genotyped four microsatellite markers to test its founder effect. It was preferable to use microsatellite markers rather than SNPs because they are more polymorphic and they provide efficient and informative genotyping as they are highly mutable often with 15 or more alleles in any populations (Baird et al., 2008). Our analysis confirmed the founder effect of the c.414G>A variant. This is the second documented evidence for a founder variant in the *CLDN14* gene. The first founder effect has been reported for the c.488C>T variant that was predominant in the island population of Newfoundland (Pater et al., 2017).

In sum, we report here the first variant responsible for hearing loss in Yemeni population. This variant is a nonsense variant located within the *CLDN14* gene and has a founder effect.

DATA AVAILABILITY STATEMENT

The datasets analyzed in this manuscript are not publicly available. Requests to access the datasets should be directed to atlili@sharjah.ac.ae.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Sharjah Research Ethics Committee (No. REC-15-11-P004). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AT designed the study. AT and AM supervised the study. WM performed the experiments and wrote the manuscript. AT, WM, and MM revised and edited the manuscript. AT, WM, MM, and SA analyzed the data. All authors have read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2019.01087/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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2.1.1 SUPPLEMENTARY MATERIALS OF ARTICLE 1

TABLE S 1. SUMMARY OF CLINICAL DATA FOR HEARING IMPAIRED IN THE THREE FAMILIES

Family	Subject	Gender	Age (y.o.)	*PTA (dB HL)	Hearing impairment	Audiogram Shape
YMN-I	YMN2	Female	25	L:60 R:80	Moderate to severe	Sloppy
	YMN3	Female	23	L:70 R:80	Severe	Sloppy
YMN-II	YMN4	Female	29	L:90 R:100	Severe-profound	Sloppy
	YMN5	Female	14	L:70 R:80	Severe	Sloppy
	YMN6	Female	21	L &R: 80	Severe	Sloppy
YMN-III	YMN8	Female	25	L &R: 100	Profound	Sloppy
	YMN9	Female	29	L:90 R:100	Severe to profound	Sloppy

*PTA: Pure-tone air-conduction average.

L: left ear, R: right ear

TABLE S 2. SEQUENCES OF PRIMERS, MELTING SIZE AND BASE PAIR SIZES

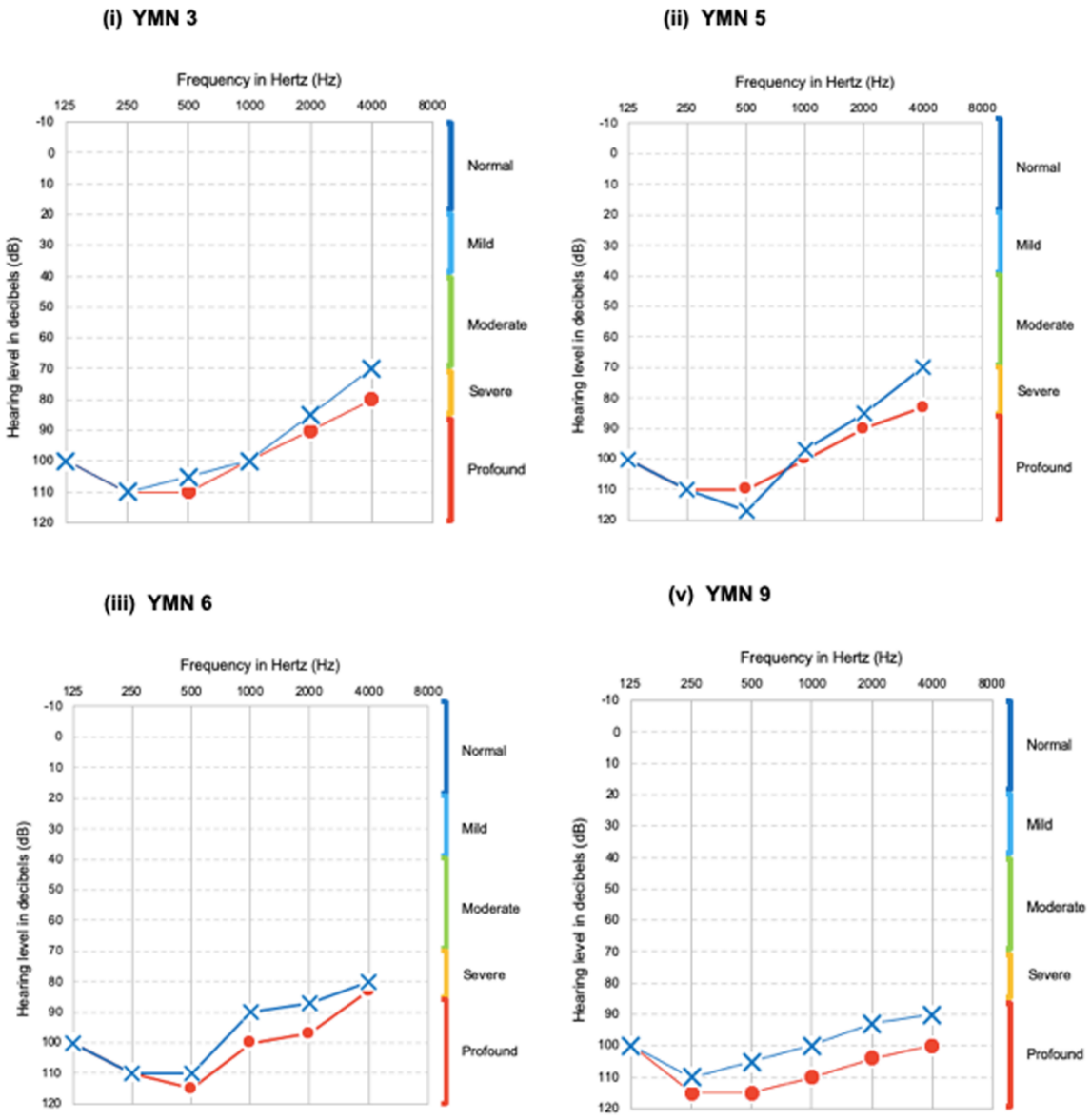
Assay	Primer name	Forward Sequence	Reverse Sequence	*Tm	Bp size
Genotyping	D21S1252	[VIC]TCTGTCTTTGTCTCACTATCTG	GCAATGCTCTGTGGCT	55°	247 bp
	D21S267	[NED]ATGGATCTGGATTTCTATCTTC	CCTCCAACCTGGGTGA	55°	114 bp
	D21S1894	[PET]ATGAGGCTCAATGCTATTGGAGTGC	AAAGCCAGCTACAAGTCTTGCTGC	55°	205 bp
	D21S168	[FAM]ATGCAATGTTATGTAGGCTG	CGGCATCACAGTCTGATAAA	55°	200 bp
Sequencing	CLDN14-seq	ACCACCATCCTGCCGCACTG	TGTTTGCAGTGGTCGTGGTG	55°	550 bp
PCR-RFLP	CLDN14	CATTTCCTTTCTCTCCCTGCT	GACATTTCCTCGCATTCACA	55°	987 bp

*****Melting temperature * BP: Base pair

TABLE S 3. MUTATIONS OF CLDN14 IN DIFFERENT POPULATIONS.

Population	Mutation	Protein	Variant	Ref
Greece and Spanish mix	c.301G>A	p.G101R	missense	[75]
India	c.254T>A	p.Val85Asp	missense	[125]
Morocco	c.11C>T	p.T4M	missense	[126]
Newfoundland	c.488C>T	p.A163V	missense	[127]
Pakistan	c.254T>A	p.Val85Asp	missense	[74]
	c.259-260TC>AT	p.Ser87Ile	missense	[67]
	c.281C>T	p.Ala94Val	missense	
	c.242G>A	p.Arg81His	missense	
	c.254T>A	p.Val85Asp	missense	
	c.398delT	p.Met133ArgfsX23	deletion	
	c.254T>A	p.Val85Asp	missense	[72]
	c.398delT	p.Met133Argfsx23	deletion	
	c.167G>A	p.w56*	nonsense	[73]
	c.242G>A	p.Arg81His	missense	
	694G>A	p.Gly232Arg	missense	
	c.254T>A	p.Val85Asp	missense	
Yemen	c.414G>A	p.Trp138Ter	nonsense	current study

Supplementary of the audiograms



Supplementary Figure 1. Audiograms for the affected individuals. (O) indicates air conduction for right ear, (X) indicates air conduction for left ear. (i) YMN3 (ii) YMN5 (iii) YMN6 and (v) YMN9.

2.2 ARTICLE 2

Mitochondrial mutations in non-syndromic hearing loss at UAE

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The mitochondrion is an important organelle that is responsible for the accessibility of power to every cell in the body, and this power comes in the form of adenosine triphosphate (ATP). As a result, the organs and tissues which require the most energy are the ones which show functional changes in mitochondrial DNA mutations (mtDNA). MtDNA mutations can cause many diseases and disorders affecting several tissues and organs in the body differently. These include nerve, muscle, endocrine, optical, and most importantly in our focus, the auditory cells. Most molecular alterations found in the mitochondrial DNA, which are associated with hearing impairment are present in *12S rRNA*, $tRNA^{Ser(UCN)}$ and the $tRNA^{Leu(UUR)}$ genes. The *12S rRNA* gene in the mitochondrial DNA (mtDNA) has been shown to be a hot spot for non-syndromic sensorineural hearing loss (SNHL). In this study, 74 out of 105 deafness individuals with no Gap Junction protein beta 2 (*GJB2*) gene selected for screening for 12s rRNA mutations. Two previous reported variants m.669T>C and m.827A>G within the 12s rRNA in two affected individuals were identified. Whole exome sequencing was performed to confirm the variants. Also, to study the effect of the mutation on the RNA structure, ViennaRNA web service and RNA draw were used which revealed that the mutation altered the secondary structure of the RNA.

To conclude, the mtDNA mutation is involved in causing SNHL in U.A.E population.

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Mitochondrial mutations in non-syndromic hearing loss at UAE

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ABSTRACT

Introduction: Hearing loss (HL) is a common sensory disorder over the world, and it has been estimated that genetic etiology is involved in more than 50% of the cases in developed countries. Both nuclear and mitochondrial genes were reported as responsible for hereditary HL. Mitochondrial mutations leading to HL have so far been reported in the MT-RNR1 gene, mitochondrially encoded 12S rRNA.
Methods: To study the molecular contribution of mitochondrial *12S rRNA* gene mutations in UAE-HL, a cohort of 74 unrelated UAE patients with no gap junction protein beta 2 (GJB2) mutations were selected for mitochondrial *12S rRNA* gene mutational screening using Sanger sequencing and whole-exome sequencing. Detected DNA variants were analyzed by bioinformatics tools to predict their pathogenic effects.
Results: Our analysis revealed the presence of two known deafness mutations; m.669T > C and m.827A > G in two different deaf individuals. Furthermore, whole-exome sequencing was done for these two patients and showed the absence of any nuclear mutations. Our study supports the pathogenic effect of the m.669T > C and m.827A > G mutations and showed that mitochondrial mutations have a contribution of 2.7% in our cohort.
Conclusions: This is the first report of mtDNA mutations in the UAE which revealed that both variants m.669T > C and m.827A > G should be included in the molecular diagnosis of patients with maternally inherited HL in UAE.

1. Introduction

Hereditary hearing loss (HL) is a heterogeneous group of disorders characterized by their clinical manifestations and modes of inheritance. Hereditary HL can be conductive, sensorineural, or a combination of both [1]; and the ratio of sensorineural hereditary HL is larger than the conductive one [2]. HL can be due to genetics and/or environmental factors and can be syndromic (~30%) or non-syndromic (~70%) [3], non-syndromic hearing loss (NSHL) can be classified by the mode of inheritance as autosomal dominant (~10–15%), autosomal recessive (~75–80%), and the remainder being X-linked, Y-linked and mitochondrial [4,5]. In cases of genetic HL; mitochondrial mutations are involved; where it is estimated to be the responsible mutation in causing approximately 5% of non-syndromic post-lingual HL and 1% of pre-lingual cases [6,7].

The clinical features of the affected individuals with mitochondrial DNA (mtDNA) disorders varies for each individual, it can be asymptotic, mild with signs limited to the extraocular and skeletal muscles and it can be severe that cause neurological complication at young age [8]. There are more than 100 pathological mutations of mtDNA that have been identified in patients with different disorders [9]. Thus, mitochondrial dysfunction affects several aspects of cell physiology and can lead to a range of human diseases, including syndromic and non-syndromic hearing loss [10]. Moreover, HL is common in patients with mtDNA disorders, however, the scope and the pathophysiology of HL is not well characterized [11]. The cochlea hair cells, the auditory sensory axis cells, stria vascularis of the cochlear duct, including the auditory neurons are metabolically active and enriched in mitochondria. Many studies showed that the cochlea defects are the main origin of the impairment with the loss of both external and internal hair cells [11,12].

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Table 1
Variants in the mitochondrial 12srRNA gene in 74 hearing impaired subjects in UAE population.

Position	Replacement	No.of subjects	Homoplasmy	Heteroplasmy	Diseases	Previously reported
669	T to C	1	+	–	Deaf	Yes
750	A to G	20	+	–	Schizophrenia -associated	Yes
827	A to G	1	+	–	Deaf	Yes
1438	A to G	32	+	+	Schizophrenia -associated	Yes

The cochlea which is a snail-shaped tube in the inner ear consumes a large amount of energy to convert sound vibrations into nerve impulses. In this process mitochondrial DNA might be involved, thus, changes in the mitochondrial DNA in the sensory hair cells of the cochlea lead to hearing loss [13,14].

As mitochondria preserve their genome; the mtDNA is distinct from the nuclear genome; this means that mitochondrial diseases can be caused by mutation in nuclear genes that encode mitochondrial proteins or by mutations in mtDNA [10]. Mutations in mtDNA can have structural and functional effects, including changes in RNA structure, reductions in the levels of mRNA or tRNA, and modification of tRNA [15, 16]. Most molecular alterations found in the mitochondrial DNA, which are associated with hearing impairment are present in genes 12S rRNA, $tRNA^{Ser(UCN)}$ and $tRNA^{Leu(UUR)}$. The 12S rRNA gene in the mitochondrial DNA (mtDNA) is a hot spot for non-syndromic sensorineural hearing loss (SNHL) [17], as some commonly reported deafness-associated mtDNA mutations including A1555G,T1095C, C1494T, mutations at position 961(T961insC, T961C and delT961C), T669C and A827G have been identified in this gene [17–26].

In this study, mutational screening of the entire mitochondrial *12S rRNA* gene was performed to determine the prevalence of mitochondrial mutations in UAE patients with NSHL. In addition, we excluded by whole exome sequencing other mutations but the mitochondrial ones in our probands, supporting the pathogenic effect of m.669T > C and m.827A > G mitochondrial mutations.

2. Materials and methods

2.1. Sample preparation

We investigated 105 sporadic unrelated patients with HL. Questionnaire and consent form were filled and signed by all participants. Saliva samples were collected from the participants using Oragene-DNA (OG-500) Kit (DNA Genotek, CANADA), and only codes were used to label DNA samples to protect the participants' privacy. Genomic DNA was extracted using the Prep IT L2P (DNA Genotek, CANADA) following

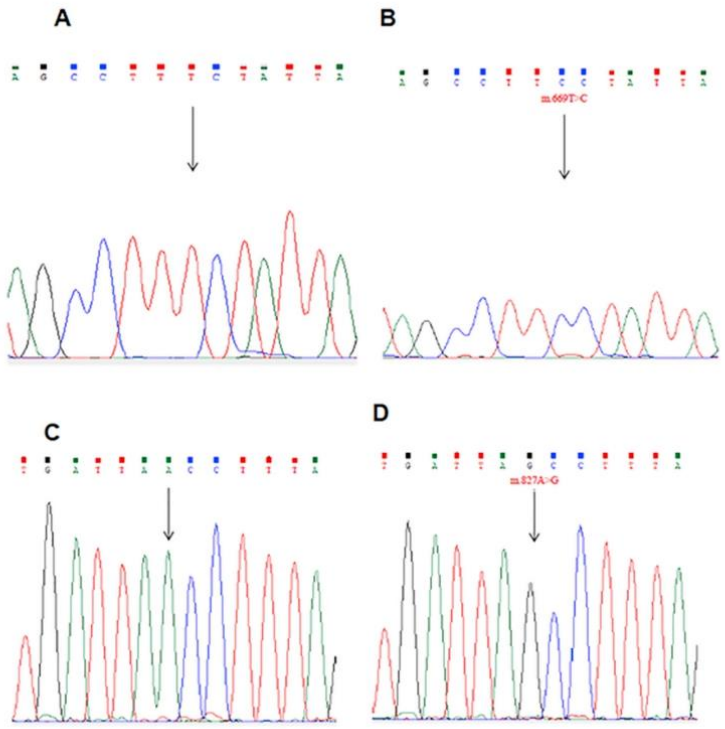

Fig. 1. Electropherogram **(A)** Normal homoplasmy individual showing the absence of m.669T > C mutation **(B)** Affected homoplasmy individual DF30 at 669 position **(C)** Normal homoplasmy individual showing the absence of m.827A > G **(D)** Affected homoplasmy individual DF31 at the position 827. Arrow indicate the mutation position.

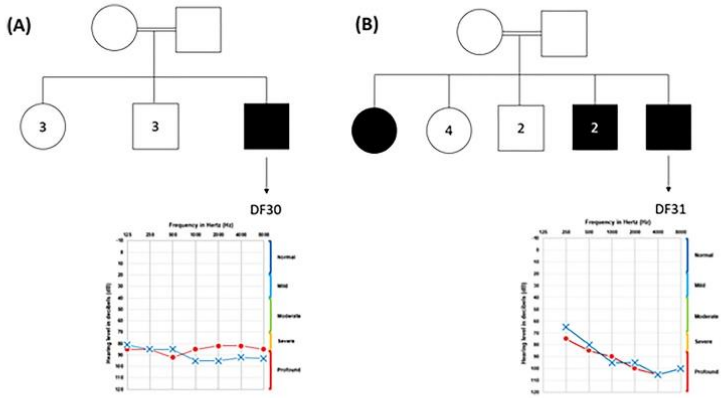

Fig. 2. Family pedigree and Audiogram. Family pedigree and audiogram of the affected individual (A) DF30, and (B) DF31. The square and circle symbols represent the male and female, respectively. The Highlighted symbols represent hearing loss patients. The double line represents consanguineous marriage. Arrow denotes the screened probands. (O) indicates air conduction for right ear; (X) indicates air conduction for left ear.

the manufacturer's protocol. The experimental procedures were approved by the Ethics Committee of the University of Sharjah (Sharjah, UAE).

2.2. PCR amplification and sanger sequencing

First, 74 out of 105 samples (age 6–45 years old) with congenital hearing loss and no *GJB2* mutations were selected. The DNA of 74 samples were amplified using two sets of 12srRNA primers: 1F (5′ CTCCTCAAAGCAATACACTG 3′) and 1R (5′ TGCT AAATCCACCTTC-GACC 3′); 2F (5′ CGATCAACCTC ACCACCTCT 3′) and 2R (5′ TGGA-CAACCAGCTATCA CCA 3′), annealing temperature for the first set of primers was 65 °C and for the second set 70 °C. For the first set of primers; bands were viewed at 833 base pair (bp) and for the second set at 801 bp.

The PCR products were purified by using enzymatic treatment Exo-SapIT clean up reagent (Applied Biosystems, USA). Big dye terminator V3.1 cycle sequencing kit (Applied Biosystems, USA) was used for performing cycle sequencing. The products were later purified using The BigDye Xterminator purification kit (Applied Biosystems, USA) following the manufacturer protocol, purified samples were loaded in a 96 well plate and injected in SeqStudio genetic analyzer (Applied Biosystems, USA).

2.3. Bioinformatics analysis

Sequences of samples were compared with the consensus Cambridge reference sequence (Gen-Bank No.NC_012920) using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov). Variants were checked using bioedit software and compared to the mitochondrial database (MITOMAP) (https://www.mitomap.org/MITOMAP) to check whether the identified variants were novel or reported. Subsequently, MITOMASTER (https://www.mitomap.org/foswiki/bin/view/MITOMASTER/WebHome) was used to check the pathogenic effect of the identified variants. Furthermore, analysis of the secondary structure of RNA was performed using ViennaRNA web services (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) and RNA draw software (https://www.rnadraw.com).

2.4. Whole exome sequencing (WES)

First, DNA was sheared and the exonic region was enriched by hybridizing to oligonucleotide capture probes (Sureselect All Exon V5+UTR). The captured fragments were adapted to produce Illumina compatible libraries and paired end sequenced (2x100 bp) on an Illumina HiSeq 2500 (Illumina, San Diego, CA, US) to produce 100X coverage. The generated sequence data have been analyzed after quality control for variant calling and annotation. Bioanalyzer plots were used at every step to assess library size and qPCR was used as well for measuring the quantity of the library before sequencing. Next, to align and recalibrate the quality of the bases; read quality was first checked and reads were aligned to GRCH37/hg19 version of the human genome and at this stage only uniquely mapped reads were selected, PCR duplicates and fragments exceeding a certain size limit were removed. Later, the filtered reads were realigned to remove false positive and misalignments. Finally, variants were called using Genome Analysis Toolkit (GATK) v2.3-9. Known variants were annotated with in-house Variation and Mutation Annotation Toolkit 2.3.4 (VariMAT). The remaining variants were then further filtered by frequency (i.e. <0.01%) in dbSNP (https://www.ncbi.nlm.nih.gov/projects/SNP/) or ExAC Browser (http://exac.broadinstitute.org/). Finally, to predict the functional impact of the candidate variant the following bioinformatics tools were used: Variant Effect Predictor (VEP) (http://grch37.ensembl.org/Homo_sapiens/Tools/VEP) and VarSome (https://varsome.com/).

Table 2
Clinical characteristic of the affected individuals.

Patient ID	Sex	Age	Number of affected siblings	Severity	Ototoxic drug	Cause of HL
DF30	Male	35	-	Profound	No	congenital
DF31	Male	39	3	Severe-profound	No	congenital

3. Results

Sanger sequencing of the *12srRNA* gene in 72 deaf individuals revealed 4 different sequence variations (Table 1), two variants were involved in deafness while two were Schizophrenia associated. The percentage of HL-related variants were 2.7% (2 out of 74). The identified variants were m.669T > G, m.827A > G detected in a homoplasmic state in DF30 and DF31 affected samples, respectively (Fig. 1).

Table 3
Variants detected by WES.

Patient ID	Gene	Position	c.DNA Variant	Amino Acid Change	Variant Type	Classification
DF30	BDP1	70805541	n.2885A > T	NA[a]	Exonic-NC[a]	Benign
	BSN	49695078	c.8089A > G	p.Lys2697Glu	Missense	Unclear
	MT-RNR1	669	n.22T > C	NA[a]	Exonic-NC[a]	Pathogenic
DF31	CLDN14	37833716	c.278T > G	p.Ile93Arg	Missense	Unclear
	OTOF	26739428	c.367G > A	p.Gly123Ser	Missense	Tolerated/Benign
	MT-RNR1	827	n.180A > G	NA[a]	Exonic-NC[a]	Pathogenic

[a] NA: Not applicable, *NC: non-coding.

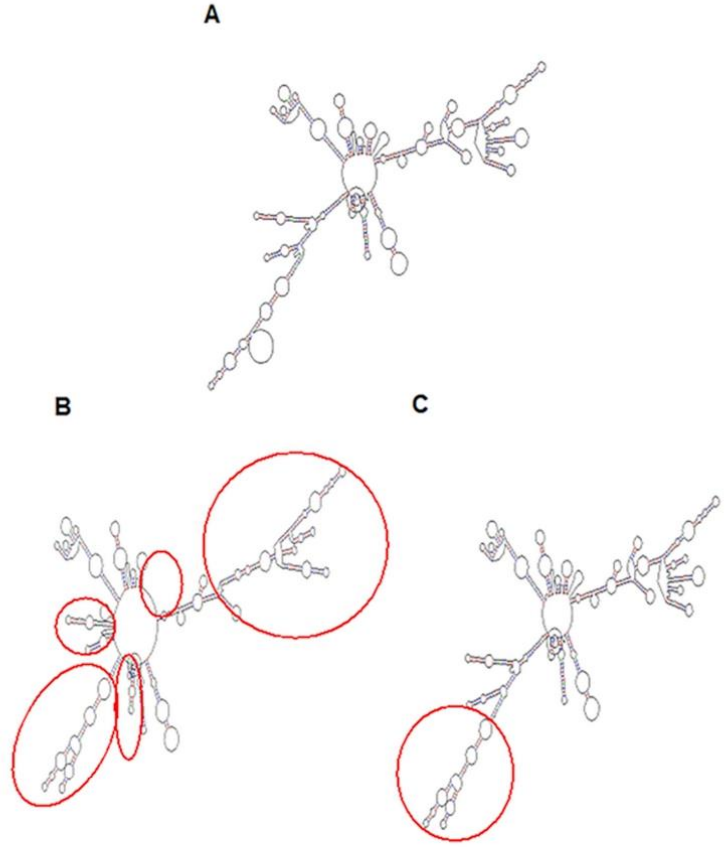

Fig. 3. RNA Secondary structure **(A)** 12s rRNA of Wildtype **(B)** 12s rRNA of affected sample DF30 **(C)** 12s rRNA of affected sample DF31. Circles indicate the changes of the 12s rRNA structure.

3.1. Clinical characteristics of DF30 and DF31 samples

Both affected individuals with mitochondrial variations (DF30 and DF31) are from consanguineous families and have congenital NSHL. Concerning DF30, the deafness was severe to profound with no affected relative (Fig. 2-A, Table 2). For DF31, the deafness was profound in both ears. Among his 9 siblings, three also were deaf: 2 brothers and one sister (Fig. 2-B, Table 2). In both families, and based on the questionnaire, environmental factors and the role of aminoglycosides in deafness were excluded (Table 2).

3.2. WES analysis

To confirm the pathogenic effect of m.669T > C and m.827A > G mutations found in two individuals (DF30 and DF31), we performed WES. The total number of variants was 356446 for the affected individual DF30 and 392247 variants were identified for the affected individual DF31. The data was filtered excluding intronic, intergenic and synonymous variant. Of the remaining variants, only variants with reading depth and allele percent equal or more than 20 were selected; in addition, only pass quality variants were kept, and only high modifier

and moderate Varimat-score were selected. After filtering the data, the variants were down to 2252 for DF30 and 1996 for DF31. Variants within deafness genes listed in hereditary hearing loss homepage (https://hereditaryhearingloss.org/recessive-genes) and MITOMAP (A Human Mitochondrial Genome Database: http://www.mitomap.org, 2019) were included (Table 3). In total, 6 variants were detected in both individuals, but only m.669T > C and m.827A > G were known to be pathogenic.

3.3. Analysis of the rRNA secondary structure

The RNA structure was predicted using RNA draw software, when the wildtype mitochondrial DNA reference sequence (NC_012920) secondary structures was compared to the affected individuals' sample (DF30 and DF31). The rRNA secondary structure for both samples were altered (Fig. 3A, B and C). This analysis showed that both mutations affect the rRNA structure. When the partial structure of the secondary rRNA for the wildtype and mutant were analyzed using ViennaRNA webservices, the position of the m.827A > G variant was changed from loop in the wildtype secondary rRNA structure to stem in the mutant secondary rRNA structure, and the position of the m.669T > C variant remained in loop in both structures (Fig. 4A and B).

4. Discussion

HL is a relatively common sensory congenital disorder, affecting approximately 120 million individuals all over the world [27] and it is one of the top severe health issues in the UAE [28]. Previous studies have shown that distinct mutations in several gene loci can cause HL in humans. In this study 74 hearing impaired affected individuals were screened with no GJB2 mutations for mtDNA mutations.

The Sanger sequencing revealed two HL-related variants in the *12s rRNA* gene. As none of the variants was reported previously in the UAE or the middle east, we decided to proceed with WES for further confirmation of their pathogenic effect. Our study showed the absence of additional HL mutations and it revealed that m.669T > C and m.827A > G is associated with HL. Because HL may result from mutations in several genes, the Next generation sequencing is preferably used for studying diseases as HL, as it enables to sequence thousands of genes in a much shorter time compared to Sanger sequencing methods, making the identification of deafness-causing variants less challenging [29,30], and allowing the detection of many causative mutations in families with HL [31].

The variants m.669T > C and m.827A > G reported in our study were previously reported in other populations summarized in Table 4. When compared the 1.3% of the m.669T > C variant detected in our study, it was slightly higher than the 1.2% detected in the Polish population, but it was less when compared to the 3.4% of the variant in the French population [24,32].

The m.669T > C variant wasn't observed in the controls in any of the previous studies and also in our present study. The frequency reported in the Human Mitochondrial Genome Database (mtDB) of the m.669T > C

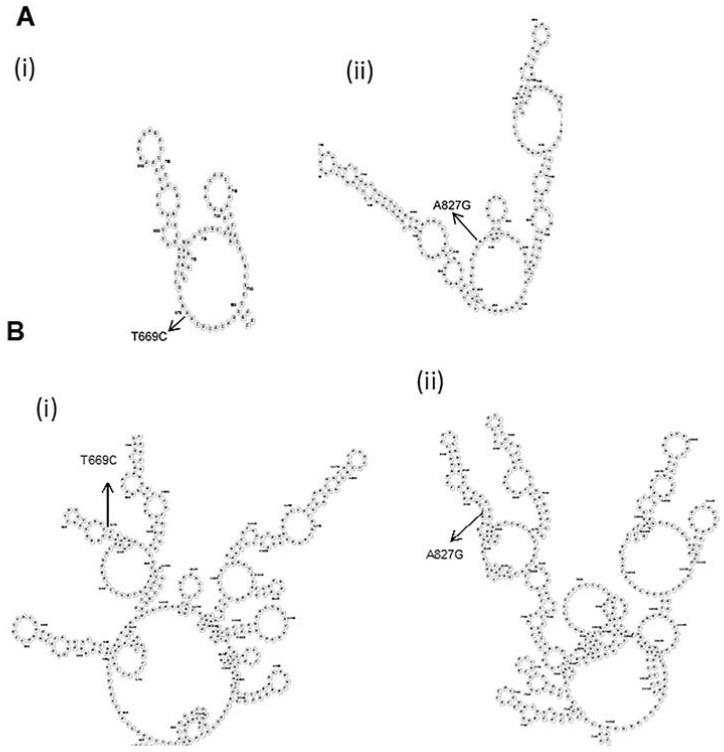

Fig. 4. Partial Structure of the secondary rRNA with variants positions **(A) Wildtype** (i) wildtype structure for sample DF30 located in loop, (ii) wildtype structure for sample DF31 located in loop **(B) Mutant** (i) mutant structure for sample DF30 located in loop, (ii) mutant structure for sample DF31 located in stem. Arrows indicated the variants found in our study.

Table 4
Frequency of the variants m.669T > C and m.827A > G reported in previous studies.

Variant	Population	No. of affected/total	References
m.669T > C	Polish	3/250	[24]
	French	1/29	[32]
	UAE	1/74	Current study
m.827A > G	Polish	1/250	[24]
	Brazil	20/152	[15]
	Brazil	5/30	[34]
	Caucasian	11/226	[26]
	Chinese	2/128	[17]
	Chinese	54/1642	[25]
	Chinese	29/662	[35]
	Chinese	6/178	[36]
	Latvian	2/191	[37]
	Iowa	17/2176	[38]
	UAE	1/74	Current study

variant is very low, 1 sequence in 2469 [33]. Moreover, the incomplete penetrance of m.669 T > C was ruled out in the Polish study [24] which may suggest that this nucleotide change is not sufficient to exhibit the clinical phenotype, as well in the France study, they stated that the number of their patient was low to rule out the pathogenicity of the m.669T > C variant [32].

Comparing the percentage of the second variant detected in our study of 1.3% with other populations, it was detected the least in the Caucasian and polish population by 0.4% [24,26], and the highest in the Brazilian population by 13.7% [15,34]. The percentage of the variant in the Chinese population was 3.79% [17,25,35,36], and finally, for the populations of Latvian and Iowa, the variant detected was almost the same by 1.05% and 1.13%, subsequently [37,38]. The m.827A > G variant was not observed in our control but was observed in the controls of Brazil study by 8.6% [15] and Chinese study by 2.7% [25]. The frequency of the variant reported in the mtDB (54/2650) suggesting, that m.827A > G may be a common polymorphic variant [33]. Moreover, the m.827A > G variant was observed in individuals with aminoglycoside exposure and/or NSHL [26,39], indicating that this mutation is involved in the pathogenesis of hearing impairment.

When comparing our result to middle east countries, none of the variants found in our study were reported, only m.1555 A > G was found within the population of Egypt and Iran [40,41] and in a minor proportion of causing NSHL in Qatar population [42], this variant was absent in our study and was not recorded before in the UAE population.

As it is very well described in biology, the structure affects the functions and might also alter the function of the protein, this might explain the change in the secondary rRNA structure of the m.669T > C and m.827A > G variants of the affected individuals with NSHL. Moreover, when comparingFigure 4-A and B, that shows the localization changes of m.827A > G variant from loop to stem, and the localization of variant m.669T > C in the loop for both wildtype and mutant structure, our data might match/together with the study of Pesini et al., when compared all the pathogenic variants in the 12s rRNA, the pathogenic mutation was higher in the stem by 92.9% than the loop which was 82.% [43]. This might rule out and confirm the pathogenic effect of the m.827A > G variants, but further investigation is required.

5. Conclusions

In conclusion, our study revealed that mitochondrial mutations are rare contributors to HL in UAE population. In addition, our findings support the pathogenic potential of the m.669T > G, m.827A > G variants as no other pathogenic variants have been detected by WES in the patients carrying these two variants, and as shown by the significant difference between the predicted secondary structure of the mutants in comparison to wildtype.

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Declaration of competing interestCOI

The authors declare no conflict of interest.

Ethic statement

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of University of Sharjah (No. REC-15-11-P004).

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3.DISCUSSION

Nowadays, hereditary hearing loss becomes the most common sensory disorder worldwide. Hereditary hearing loss and deafness may be considered syndromic or non-syndromic. Syndromic hearing impairment is associated with external ear malformations, malformations in other organs, or medical problems involving other organ systems. Non-syndromic hearing disorder has no associated apparent external ear defects or any related medical problems; however, it may be associated with middle ear and/or inner ear abnormalities. Approximately 80 percent of prelingual deafness is inherited, predominantly autosomal recessive and non-syndromic [43].

Autosomal recessive nonsyndromic hearing loss (ARNSHL) is a monogenic disorder with a high genetic heterogeneity. Mutation screening of common recessive genes , predominant genes or even genes that can be recessive and dominant as GJB2 [128] and TMC1 [129] by conventional methods such as PCR and Sanger sequencing can be sometimes inefficient to identify the responsible mutation in unclassified affected [130,131]. Also, the platform hereditaryhearingloss.org is updated to date with a list of 76 genes associate with ARNSHL (**Annex table 2**), and the conventional way to screen those panel of genes is time-consuming and expensive. Next Generation Sequencing (NGS) is a very effective approach [132] for screening high heterogeneity diseases, such as hearing loss, with more than hundreds of genes and large common mutations [133], it has the ability to sequence thousands of genes in much shorter time compared to Sanger sequencing method. NGS can identify many causative mutations in small families with HL [134,135].

Many reports claim that approximately 50% of autosomal recessive deafness is caused by either homozygous or compound heterozygous mutation in the *GJB2* gene [136]. Therefore, to identify a new variant mutations located in *CLDN14* (**Article 1**), mtDNA (**Article 2**) and *TMC1* (**Annex 5.2**) gene, we first confirmed the absence of mutations in the *GJB2* gene as it is reported the most common mutation in the variant 35delG as described in many parts of the world identified as responsible for deafness in previous studies [137,138].

In **article 1**, we analyzed three Yemeni families with ARNSHL. The clinical exome sequencing (CES) was done for the affected individuals in the three families. The CES approach of NGS revealed the novel variant p.Trp138Ter (c.414 G>A), which the underlying cause of HL among these individuals. The CES was selected in this study because it is an advanced method for

the detection of disease-causing DNA variants within 1% of the genome that codes for proteins (exons) or flanks regions that code for proteins (splice junctions) and it has been rapidly extended to the discovery of variants in research settings. Further, recent improvements in precision have enabled the development of CES for mutation detection in patients with suspected genetic diseases [145]. The nonsense variant p.Trp138Ter (c.414 G>A) found in this study was not reported before, another nonsense variant at a different position; c.167G>A (p.Trp56Ter) was reported in Pakistani families [87]. Mostly, the previously reported variants within the *CLDN14* gene are missense mutations which are shown in Table S 3. Mutations of CLDN14 in different populations.in **Article 1**. Also, CLDN14 mutations have not been previously associated with hearing loss in the middle east or in approximate geological regions to Yemen. Moreover, the CLDN14 was previously reported in Canada [127], Pakistan [67,72–74], and India [125] as the causative gene responsible for hearing loss. In addition, a study in Morocco identified CLDN14 mutation among 80 families and was reported as a causative mutation in the population [126], these findings show that CLDN14 variants are distributed widely in different ethnic populations, but linking its function to hearing loss and phenotypic variability may vary in different populations due to the diverse genetics or environmental factors [131].

The main function of CLDN14, was first discovered through studies in consanguineous families from the genetically isolated population of Pakistan [67]. Since c.414G > A was observed in three different families, we genotyped four microsatellite markers to test its founder effect. It was preferable to use microsatellite markers rather than SNPs because they have a high heterozygosity and have high genotyping efficiency in any genetic or population analysis, as they are also highly mutable markers with 15 or more alleles in any population [139]. Our study confirmed the founding effect of the variant c.414G > A. This is the second known proof of a founder mutation in the CLDN14 gene. The first founder effect was recorded for the variant c.488C > T in the island population of Newfoundland [127].

The audiograms of the affected individuals indicated that individuals with CLDN14 mutations can have various degrees of hearing loss and that the loss is greater at high frequencies, as a consequence of the absence or the incomplete translated protein due to the presence of the c.414G>A mutation. The deafness phenotypic variability observed in our study has been also reported in different families with *CLDN14* mutations. As demonstrated in the previous study in Pakistani population [74], when comparing audiograms for affected individuals, different levels

of affectivity of the CLDN14 mutation to the hearing frequency were reported from mild to extreme, from moderately severe to profound, and the highest frequencies ranged from severe to profound. A milder type of hearing loss due to mutation in CLDN14 has also been recorded previously in another study in the Pakistani population [67].

As our research focused on identifying the genes and mutations responsible for hearing loss in the Arab population, the second study was performed in the United Arab Emirates (U.A.E). In **Article 2**, only 74 of the 105 affected individuals were identified without a GJB2 mutation and screened using Sanger sequencing. Sanger sequence method identified two hearing loss-related variants in the 12s rRNA gene. The two variants m.669T > C and m.827A > G in the 12s rRNA gene have not been found before in the U.A.E. or any Arab country as a result of which we have chosen to continue with the whole exome Sequencing (WES). Also, we decided to proceed with this method because the WES, which is an approach of NGS, allows variations in the protein-coding region of any gene to be detected, rather than in a few selected genes. Since most known mutations that cause disease occur in exons, WES is thought to be an effective tool for detecting potential disease-causing mutations. The WES confirmed the absence of other genes related to HL in both affected individuals and only the two variants m.669T>C and m.827A>G were associated with HL.

The variants reported in our study have previously been reported in studies in Europe, Asia, North America, and the United States [149–157]. Variant m.669T > C was reported only in two European countries (France and Poland) [140,141], it was higher in the French population at 3.2% [140] compared to our study at 1.3% and slightly lower in the Polish population at 1.2% [141]. Variant m.837A > G was reported in more populations compared to m.669T > C, the highest in the Brazilian population was reported by 13.7% [142,143] and the lowest in the Caucasian and Polish population by 0.4% [141,144]. The number of variants in the Chinese population was 3.79 % [145–148] and, finally, for the Latvian and Iowa populations, the variant was found to be almost the same by 1.05% and then by 1.13 % [115,116]. Since both variants implicated in aminoglycoside -induced hearing loss [141,149,150], clinical characteristic of both affected individuals was analyzed, and there was no exposure to aminoglycoside antibiotics and no other symptoms associated with the HL.

As well defined in biology, the structure influences the functions and may also modify the function of the protein, this may explain the shift in the secondary rRNA structure of the m.669T>C

and m.827A > G variants of the affected individuals with NSHL. In addition, when the ViennaRNA web services was used to further study the secondary structure of the rRNA, changes in the position from the loop to the stem was seen in the m.827A > G variant confirming the pathogenicity of the variant. This was also demonstrated in the Pesini et al. study, when all pathogenic variants were compared in 12s rRNA, 92.9% higher in the stem than 82% higher in the loop. [151].

Besides the CLDN14 and mtDNA genes, the *TMC1* gene was also found to cause HL in Arabs. In **annex 5.2**, we found a nonsense mutation in the *TMC1* gene in two families. This mutation corresponds to the transition from T to C at position 100 of the coding region (c.100 C > T) and is located in exon 7, replacing Arginine 34 with a stop codon (p. Arg34X). The variant accounts for more than 30% of TMC1 mutant alleles and reported in populations in Asia and North Africa [125,131,135].

TMC1 mutations that account for hearing loss phenotypes in 3–6 % of families are a common cause of ARNSHL in India, Pakistan, Tunisia, and Turkey. High rates of consanguinity and endogamy result in high carrier frequencies for recessive mutations [163], thus retaining the haplotype flanking these mutations [135,163].

4. CONCLUSION

Because of the complexity of the mutations causing HL and the lack of information on the HL biomarkers specific to the Arab and UAE populations, we designed our study to identify new genetic variants using whole-and target exome sequencing using the NGS. In this thesis, the following significant findings are presented:

1. Due to consanguineous marriages in the Arab populations, inherited HL is high and the probability of detecting novel mutations is also high.
2. The nonsense mutation within the *CLDN14* gene found in the Yemeni families is contributed to HL and has a founder effect.
3. When screening for novel mutations in the Yemeni population, the genes *GJB2* and *CLDN14* may be examined first to confirm both genes' absence.
4. To date, we are the first to report HL- a related study in the Yemeni population.
5. Our analysis showed that mitochondrial mutations are rare contributors to HL in the UAE population.
6. Our results support the pathogenic potential of m.669 T > G, m.827A > G variants. The WES did not identify other pathogenic variants in patients carrying these two variants.
7. The difference between the mutants' predicted secondary structure and the wild type confirmed that the mutation is pathogenic and affected the 12S rRNA secondary structure.
8. The clinical exome sequencing, Sanger sequencing, and RFLP-PCR identified mutation p. Arg34X in two different families. This indicates that it is a major contributor to DFNB7/11 type of deep deafness of the UAE population.
9. Finally, the thesis findings can help in genetic counseling, prenatal screening and postnatal genetic diagnosis at the Arab population.

5.ANNEX

5.1 TABLES

TABLE 2. GENES ASSOCIATED WITH AUTOSOMAL DOMINANT NONSYNDROMIC HEARING LOSS.

Gene	Locus	Onset	Gene	Locus	Onset
ACTG1	DFNA20/26	Post-lingual	*MYH9*	DFNA17	Prelingual
CCDC50	DFNA440	Post-lingual	*MYO3A*	DFNB30	Post-lingual
CD164	DFNA66	Post-lingual	*MYO6*	DFNA22	Post-lingual
CEACAM16	DFNA4B	Post-lingual	*MYO7A*	DFNA11	Post-lingual
COCH	DFNA9	Post-lingual/2nd	*NLRP3*	DFNA34	Post-lingual
COL11A1	DFNA37	Post-lingual/2nd	*OSBPL2*	DFNA67	Post-lingual
COL11A2	DFNA13	Post-lingual/2nd	*P2RX2*	DFNA41	Post-lingual/2nd
CRYM	DFNA40	Prelingual	*PDE1C*	DFNA74	Postlingual/3rd
DIAPH1	DFNA1	Post-lingual/1st	*PLS1*	DFNA76	Post-lingual
DMXL2	-	Post-lingual/2nd	*POU4F3*	DFNA15	Prelingual/Post-lingual
DSPP	DFNA39	Post-lingual	*PTPRQ*	DFNA73	Post-lingual
EYA4	DFNA10	Post-lingual/3rd, 4th	*REST*	DFNA27	Post-lingual
GJB2	DFNA3	Prelingual	*SCD5*	-	Post-lingual
GJB3	DFNA2B	Post-lingual/4th	*SIX1*	DFNA23	Postlingual/1st
GJB6	DFNA3	Prelingual	*SLC12A2*	-	Post-lingual
GRHL2	DFNA28	Post-lingual	*SLC17A8*	DFNA25	Prelingual/post-lingual 1st
GSDME/ DFNA5	DFNA5	Post-lingual	*SMAC/DIABLO*	DFNA64	Post-lingual
HOMER2	DFNA68	Post-lingual/1st	*TBC1D24*	DFNA65	Post-lingual
IFNLR1	DFNA2C	Post-lingual	*TECTA*	DFNA8/12	Post-lingual
KCNQ4.	DFNA2	Post-lingual/2nd	*TJP2*	DFNA51	-
KITLG	DFNA69	Post-lingual	*TMC1*	DFNA36	Post-lingual
LMX1A	DFNA7	Prelingual/Post-lingual	*TNC*	DFNA56	Post-lingual
MCM2	DFNA70	Post-lingual	*TRRAP*	DFNA75	Post-lingual
MIRN96	DFNA50	Post-lingual/2nd	*WFS1*	DFNA6/14/38	Post-lingual
MYH14	DFNA4A	Post-lingual			

TABLE 3. GENES ASSOCIATED WITH AUTOSOMAL RECESSIVE NONSYNDROMIC HEARING LOSS.

Gene	Locus	Onset	Gene	Locus	Onset
ADCY1	DFNB44	Prelingual	*MYO3A*	DFNB30	Prelingual
BDP1	DFNB49	Post-lingual	MYO6	DFNB37	Prelingual
BSND	DFNB73	Prelingual	MYO7A	DFNB2	Prelingual
CABP2	DFNB93	Prelingual	NARS2	DFNB94	Prelingual
CDC14A	DFNB105	Prelingual	OTOA	DFNB22	Prelingual
CDH23 [1]	DFNB12	Prelingual	OTOF	DFNB9	Prelingual
CIB2	DFNB48	Prelingual	OTOG	DFNB18B	Prelingual
CLDN14	DFNB29	Prelingual	OTOGL	DFNB84	Prelingual
CLDN9	-	Prelingual	PCDH15	DFNB23	Prelingual
CLIC5	DFNB103	Prelingual	PDZD7	DFNB57	Prelingual
COL11A2	DFNB53	Prelingual	PJVK	DFNB59	Prelingual
DCDC2	DFNB66	Prelingual	PNPT1	DFNB70	Prelingual
ELMOD3	DFNB88	Prelingual	PPIP5K2	DFNB100	Prelingual
EPS8	DFNB102	Prelingual	PTPRQ	DFNB84	Prelingual
EPS8L2	-	Post-lingual	RDX	DFNB24	Prelingual
ESPN	DFNB36	Prelingual	ROR1	DFNB108	Prelingual
ESRRB	DFNB35	Unknown	S1PR2	DFNB68	Prelingual
FAM65B	DFNB104	Prelingual	DFNB32	DFNB105	Prelingual
GAB1	DFNB26	Prelingual	SERPINB6	DFNB91	Prelingual
PJVK	DFNB59	Prelingual	SLC22A4	DFNB60	Prelingual/ Post-lingual
GIPC3	DFNB15/72/95	Prelingual	SLC26A4	DFNB4	Prelingual
GJB2	DFNB1	Prelingual	SLC26A5	DFNB61	Prelingual
GJB6	DFNB1	Prelingual	SPNS2	-	Prelingual
GPSM2	DFNB32/82	Prelingual	STRC	DFNB16	Prelingual
GRXCR1	DFNB25	Prelingual	SYNE4	DFNB76	Prelingual
GRXCR2	DFNB101	Prelingual	TBC1D24	DFNB86	Prelingual
HGF	DFNB39	Prelingual	TECTA	DFNB21	Prelingual
ILDR1	DFNB42	Prelingual	TMC1	DFNB7/11	Prelingual
KARS1	DFNB89	Prelingual	TMEM132E	DFNB99	Prelingual
LHFPL5	DFNB67	Prelingual	TMIE	DFNB6	Prelingual
LOXHD1	DFNB77	Post-lingual	TMPRSS3	DFNB8/10	Prelingual/post-lingual
LRTOMT/COMT2	DFNB63	Prelingual	TPRN	DFNB79	Prelingual
MARVELD2	DFNB49	Prelingual	TRIOBP	DFNB28	Prelingual
MET	DFNB97	Prelingual	TSPEAR	DFNB98	Prelingual
MPZL2	DFNB111	Prelingual	USH1C	DFNB18	Prelingual
MSRB3	DFNB74	Prelingual	WBP2	-	Prelingual
MYO15A	DFNB3	Prelingual	WHRN	DFNB31	Prelingual

5.2 ARTICLE

Identification of nonsense mutation in *TMC1* gene inducing hearing loss by clinical exome sequencing

M.Alobathani, A.Al Mutery, W.K.E. Mohamed and A. Tlili

TMC1 is the sixth most common cause of recessive HL in the world and one of the most common causes of ARNSHL in consanguinity belt populations. TMC1 mutations are a common cause of ARNSHL in many countries like India, Iran, Pakistan, Tunisia, and Turkey, where 3–6% of families account for hearing-loss phenotypes [143–145]. The most common HL recessive mutation in the TMC1 gene is p. R34X (c.100C > T) and has been shown to have originated from two founders [143]. It accounts for more than 30–40% of the mutant alleles of TMC1 and occurs in populations in the Middle East and North Africa [144], such as Pakistan, Saudi Arabia, Turkey, and Tunisia. The variant (p. R34X) so far was not reported in the UAE population, but another variant in the TMC1 (p. Arg34X) was only reported in UAE which was not reported in other Arab countries.

In this study, the variant p. Arg34X was identified with the help of NGS techniques using clinical exome sequencing and the variant was confirmed using sanger sequencing and PCR-RFLP. Using bioinformatics tools confirmed the variant as disease-associated with HL.

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Identification of nonsense mutation in *TMC1* gene inducing hearing loss by clinical exome sequencing

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ABSTRACT

Hearing loss is one of the most common sensorineural disorders, affecting one in 1000 individuals that can be classified into syndromic and non-syndromic. *TMC1* gene has been identified as a non-syndromic gene for both autosomal and recessive forms. In this study, UAE consanguineous family with congenital profound non-syndromic hearing loss was characterized. By using clinical exome sequencing, Sanger sequencing and PCR-RFLP, the p.Arg34X as the disease-associated variant was identified. The screening of other families with deafness revealed the presence of this nonsense mutation in one additional family and suggested that p.Arg34X is major contributor to DFNB7/11 form of deafness in UAE population. The present investigation reports to be the first study associating *TMC1* mutations to hearing loss in the GCC region.

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Introduction

Hearing loss is one of the most common sensorineural disorders, affecting one in 1000 individuals; and can be classified into syndromic and non-syndromic forms. Approximately, 80% of hearing loss is non-syndromic and implicates more than 100 genes (Egilmez et al. 2016). Almost, 70% of mutations responsible for non-syndromic hearing loss (NSHL) are autosomal recessives (Belguith et al. 2009). Today, 108 loci associated with autosomal recessive non-syndromic hearing loss (ARNSHL) have been reported and 71 genes have been identified (Tlili et al. 2017b).

Mutations in the *TMC1* gene are responsible for both dominant and recessive NSHL (DFNB7/11 and DFNA36). In 2002, Kurima et al., narrowed the critical overlapping interval between DFNB7/11 and DFNA36 to 3 Mb. The bioinformatics analysis of the candidate region and Sanger sequencing of a new gene (*TMC1*) in the affected members DFNA36 and DFNB7/11, revealed the presence of mutations in the new gene *TMC1*. This gene is one of the transmembrane channels-like (*TMC*) genes that share a conserved 120 amino-acids (*TMC*

domain) of unknown function (Kurima et al. 2002a). In the cochlea, *TMC1* is required for the normal function of mechanically-activated channels (Kawashima et al. 2015; Pan and Holt. 2015; Maeda et al. 2014; Pan et al. 2013). The hearing defect has also been reported in *Tmc1* mutant mice *dn* (deafness) and *Bth* (Beethoven) (Keats et al. 1995; Vreugde et al. 2002). Electronic microscopy studies of both animal models showed a degeneration of cochlear hair cells (Vreugde et al. 2002).

TMC1 gene is one of the five major genes causing profound recessive deafness worldwide (Kitajiri et al. 2007; Kurima et al. 2002b; Riazuddin et al. 2012; Yang et al. 2013). Mutations in this gene account 3% to 6% of deafness in Turkish Tunisian, European, Indian and Pakistani populations (Kalay et al. 2005; Tlili et al. 2008; Schrauwen et al. 2013). In the present study implication of *TMC1* gene in two different consanguineous families from UAE were described for the first time.

Materials and Methods

Family recruitment and DNA extraction

In this study, two UAE families with ARNSHL were recruited. Clinical examinations and analysis excluded environmental factors and other symptoms. Informed consent from all participants and parents of subjects younger than 18 years were obtained before sample (saliva) collection. Genomic DNA has been extracted by Oragene-DNA kit (DNA Genotek, Canada). In total, DNA has been extracted from 8 family members, 92 unrelated deaf patients and 120 healthy individuals from UAE population. All experiments were already approved by the University of Sharjah Ethics Committee.

Clinical exome analysis

After checking the quality control for the DNA, capture-based method was used to prepare the clinical exome sequencing (CES) library. Biotinylated oligonucleotide capture probes (Roche Life sciences) also called as baits that were designed for the exonic regions of ~ 6800 clinically relevant genes were used to enrich by hybridization. Qubit High Sensitivity reagent was used to quantify that prepared libraries. The obtained libraries were diluted to final concentration of 2nm in 10 ul and were subjected for Cluster amplification. Once the cluster generation was completed, the flow cells were loaded on to the sequencer. Hi Seq X ten was carrying the sequencing results to generate 2X150 bp sequence reads at 100X sequencing depth (~4 GB). Sequenced data were processed to generate FASTQ files and uploaded on the FTP server for download. Data received from CES was compressed in BAM files that was processed by SAM tools (samtools-1.2), as well hg19/GRCh37 was built by mapping the paired-end (2x100 bases) DNA sequence reads that proceed the quality control to the human reference genome by BWA tools (bwa-0.7.12). Analyzing data from BAM files, to identify SNP/point mutations and short indels in the exome samples, realignment and recalibration were performed using Genome analysis toolkit (GATK) (v2.3-9). To rise the accuracy with a more precession, different variants were filtered according to specific sequencing depth (100b) with the minimum reads =10b.

Sanger sequencing

To sequence *TMC1* gene with exon 7, the following primers were designed: Forward 5'CACGATGTGGAGAATTGCTAGA3' and Reverse 5'GCATCATCAGATTAAGGCTCTC3'. Wizard SV Gel was used to purify the PCR products. Big Dye Terminator V3.1 cycle sequencing kit and the genetic analyzer ABI 3500 genetic analyzer (Applied Biosystems, Thermo Fisher Scientific, USA) were used to sequence PCR products.

PCR-RFLP

In order to check the segregation of the c.100 C>T mutation in the two affected UAE families, and to screen 92 unrelated deaf patients and 120 healthy individuals, the *TaqI* restriction enzyme has been used. PCR reaction products were digested according to manufacturer's instructions

(New England Biolabs, USA) and run on agarose 2% gel.

Results

In this study, one UAE consanguineous family with ARNSHL was recruited. Audiogram analysis showed that both affected individuals have profound deafness (Figure 1). First *GJB2* gene mutations which are the most common deafness mutations in UAE population were analyzed (Tlili et al. 2017a), and absence of the *ILDR1* mutations previously reported in one UAE deaf family were verified (Tlili et al. 2017b). These analyses showed the absence of pathogenic variants in the tested chromosomal regions. Next, target genomic capturing and clinical exome sequencing for a panel of ~6800 genes including 124 genes related to hereditary deafness, was conducted using the DNA from the proband (Fig 1).

The clinical exome analysis using the proband's DNA revealed 407727 variants. To determine the causing variant, several filtrations were performed like: i) only variants located within genes responsible for deafness were considered, ii) as the family is consanguineous, only homozygous variants were kept, and iii) variants with a frequency more than 0.01 were removed. This filtration resulted in only 126 candidate DNA variations: 3 in UTR regions, 122 intronic and one nonsense (Table 1). The last one was located in the *TMC1* gene (c.100C>T) and it substitutes the Arginine amino acid at position 34 into a stop codon (p.Arg34X) (Table 1). As this variant has been associated with deafness in previous studies, it corresponds to the responsible mutation for ARNSHL observed in the proband. This finding has been confirmed by Sanger and RFLP analyses that showed the co-segregation of the c.100C>T mutation with deafness in the proband's family (Fig 1 & 2). The screening of additional 93 deaf and 120 control individuals revealed the presence of this mutation in one unrelated patient with deafness. The co-segregation of c.100C>T mutation with the disease in this patient's family has been confirmed by PCR-RFLP. Obtained data suggested that this mutation has a frequency of 2% in the UAE individuals with ARNSHL.

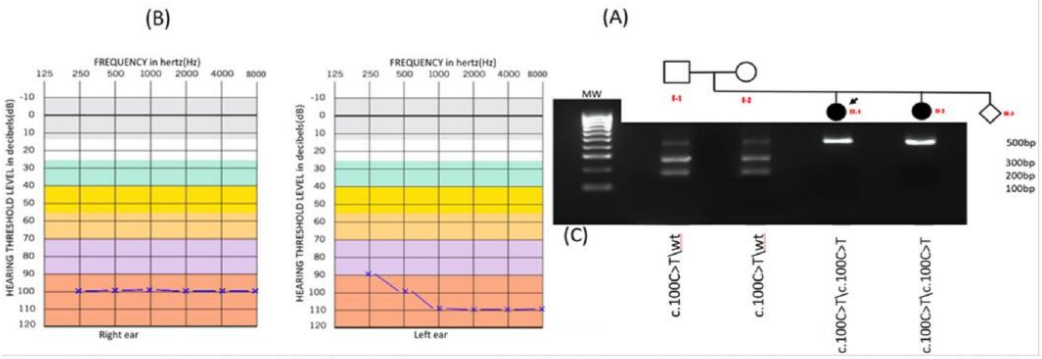

Fig 1. Pedigree of the affected family, Audiogram and PCR-RFLP analysis. (A) Pedigree of the affected family with non-syndromic hearing loss. Arrow denotes the proband. (B) Audiogram of the proband individual shows a profound sensorineural hearing loss. (C) Results of PCR-RFLP analysis of DNA of the affected family with non-syndromic hearing loss. A 427 bp PCR fragment is digested with Taq1 restriction enzyme. The wild-type DNA is cleaved into three fragments 264 and 163 bp, whereas the c.100C>T mutant allele is not cleaved. MW: DNA Ladder (100bp DNA Ladder, REF G2101).

Table 1: Filtration results of the 407727 variants obtained by the clinical exome sequencing.

Variant Class	Gene Name	c.DNA variants
3UTR	ADCY1 (+)	(c.*4682G>A)
	DCDC2 (-)	(c.*884A>T)
	TMEM132E (+)	(c.*34_*35insA)
Intronic	36 genes	122 variants
Nonsense	TMC1 gene	(c.100C>T)

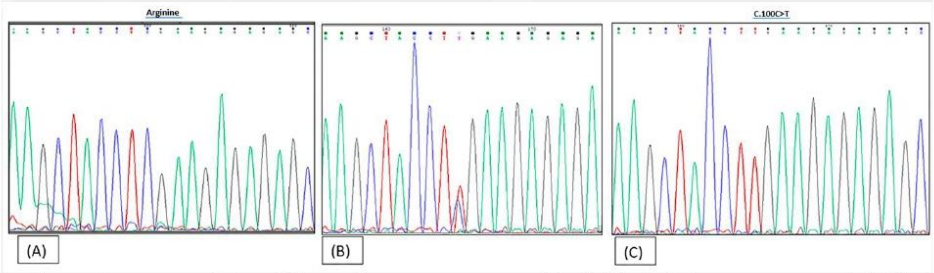

Fig 2. Electropherograms. (A) Wild-type homozygous normal individual, (B) Heterozygous individual. (C) Affected individual with the c.100C>T pathogenic variant in the *TMC1* gene (NC_000009.12).

Discussion

ARNSHL is a monogenic disorder with a high genetic heterogeneity. Mutation screening of common variants and predominant genes by classical and conventional methods can be sometimes efficient to identify the responsible mutation in unclassified affected individuals (Lu et al. 2018). However, to screen the total of 71 genes associated today with ARNSHL is time-consuming and expensive (Wang et al. 2018). Therefore, it was confirmed that the absence of mutations in the GJB2 gene and the *ILDR1* mutation c.804delG reported in previous studies in UAE population (Tlili et al. 2017b, Tlili et al. 2017a), finally summarizing a clinical exome of ~6800 genes including 124 deafness-related genes. Hence, homozygous nonsense mutation in the *TMC1* gene of the proband was identified which is corresponds to a transition T to C at position 100 of the coding region (c.100 C>T) substituting the Arginine 34 by a stop codon (p.Arg34X). This pathogenic variant has been previously reported in several populations with different frequencies and it was characterized by a marker founder effect (Kurima et al. 2002b,Kitajiri et al. 2007,Hilgert et al. 2008,Tlili et al. 2008,Ben Said et al. 2010,Sirmaci et al. 2009). In the present study, mutation was found in an additional UAE family and suggested that it contributes to 2% of ARNSHL in UAE population. Moreover, the audiogram revealed profound hearing loss similar to previous phenotypes associated to this mutation in many others populations (Scott et al. 1996; Tlili et al. 2008; Kitajiri et al. 2007; Sirmaci et al. 2009; Kurima et al. 2002a) which confirmed the deleterious effect of the p.Arg34X mutation.

Conclusion

To conclude, this study reports for the first time an association between *TMC1* gene mutations and ARNSHL in UAE and the GCC region. The identified mutation p.Arg34X has been detected in two different families, which suggests that it can be a major contributor to DFNB7/11 form of deafness in the UAE population.

Conflict of interest

There is no conflict of interest.

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5.3 DEAFNESS RELATED ARTICLES.

The articles present below were published while this thesis was in execution.

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ORIGINAL ARTICLE

Prevalence of *GJB2* Mutations in Affected Individuals from United Arab Emirates with Autosomal Recessive Nonsyndromic Hearing Loss

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Aim: Mutations in the gap junction protein beta 2 (*GJB2*) gene are responsible for more cases of nonsyndromic recessive hearing loss than any other gene. The purpose of our study was to evaluate the prevalence of *GJB2* mutations among affected individuals from United Arab Emirates (UAE).
Methods: There were 50 individuals diagnosed with hereditary hearing loss and 120 healthy individuals enrolled in the study. The Sanger sequencing method was used to screen the *GJB2* coding region in all affected individuals. The c.-1G>A variant was determined by the polymerase chain reaction-restriction fragment length polymorphism method in normal individuals.
Results and Discussion: Nine cases with bi-allelic mutations and three cases with mono-allelic mutations were detected in 12 out of 50 patients (24%). The homozygous mutation c.35delG was identified as the cause of hearing loss in six participants (12%). The mutation c.506G>A was identified in three affected individuals (6%). The allelic frequency (14%) and low ratio of individuals homozygous (12%) and heterozygous (2%) for the c.35delG mutation suggest that there are other genes responsible for nonsyndromic deafness in the UAE population. The results reported here are a preliminary step in collecting epidemiological data regarding autosomal recessive nonsyndromic hearing loss related to *GJB2* gene mutations among the UAE population.
Conclusion: The c.35delG mutation of the *GJB2* gene is the most frequently seen causative mutation in the UAE and is followed by p.Cys169Tyr mutation.

Keywords: hereditary hearing loss, prevalence, *GJB2* mutations

Introduction

HEARING LOSS, the most common sensory defect, affects ~1 in 1000 newborns (Morton, 1991). More than 50% of all cases of hearing loss have a genetic etiology (Marazita *et al.*, 1993). Genetic forms can be classified into syndromic (hearing loss associated with other symptoms) or non-syndromic. The genetic transmission of nonsyndromic hearing loss is autosomal recessive in 80% of cases, and autosomal dominant in 20% of cases. X-linked (1%) and mitochondrial (<1%) forms have also been reported (Morton, 1991).

To date, 105 nonsyndromic recessive loci have been mapped and 64 deafness genes have been identified (Hereditary Hearing Loss Homepage: http://hereditaryhearingloss.org). Among these genes, gap junction protein beta 2 (*GJB2*) gene, located on chromosome 13, represents a major genetic cause of nonsyndromic hearing loss in several populations worldwide (Chan and Chang, 2014). The prevalence of *GJB2* mutations is high in Europe (27.1%) (Chan and Chang, 2014) and low in three Gulf Cooperation Council (GCC) studied countries: Qatar (3.2%), Oman (0%), and Kingdom of Saudi Arabia (KSA) (6.7%) (Simsek *et al.*, 2001; Al-Qahtani *et al.*, 2010; Khalifa Alkowari *et al.*, 2012).

To date, >90 deafness mutations responsible for autosomal recessive nonsyndromic hearing loss (ARNSHL) have been described in the *GJB2* gene (The Connexin-deafness Homepage: http://davinci.crg.es/deafness). One particular mutation, c.35delG, a truncating mutation, accounted for the majority of *GJB2* mutations detected in the Mediterranean populations (Lucotte, 2007). In other populations, this mutation is less frequent or absent, and sometimes other frequent pathogenic variations are present. These include c.235delC in East Asia (Fuse *et al.*, 1999), p.V37I in Southeast Asia (Kelley *et al.*, 1998), p.W24X predominant on the Indian

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Table 1. *GJB2* Allelic Distribution Detected in United Arab Emirates Patients

Allele	*No. of alleles*	*Percentage of allele (%)*	*Percentage of patients (%)*	*Type of variation*	*Effect*
c.-1G>A	1	1	2	Transition	Pathogenic
c.35delG	14	14	16	Frameshift	Pathogenic
c.506G>A (p.Cys169Tyr)	6	6	6	Missense	Pathogenic
c.438 C>T (p.Phe146Phe)	2	2	4	Silent	Unknown
c.-22-12C>T	1	1	2	Transition	Benign

GJB2, gap junction protein beta 2.

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subcontinent (Kelsell *et al.*, 1997), c.167delT in Ashkenazim (Zelante *et al.*, 1997), and p.R143W in Ghanaians (Brobby *et al.*, 1998). Moreover, one particular variation p.Cys169Tyr (c.506G>A) first described as a benign variant (Khalifa Alkowari *et al.*, 2012) has been identified as causative of recessive nonsyndromic hearing loss in two Qatari families (Birkenhager *et al.*, 2014; Zonta *et al.*, 2015).

Until now, no mutational analysis of the *GJB2* gene in United Arab Emirates (UAE) patients with ARNSHL has been performed. To provide accurate genetic testing and counseling, 50 patients with ARNSHL were recruited. Both *GJB2* gene exons were sequenced and analyzed.

Materials and Methods

Patients

This study included 50 unrelated patients (males/females 39/11) who were admitted to the UAE deaf association. The mean age was 33 (range 8–45) years. Clinical examination and evaluation revealed nonsyndromic bilateral severe sensorineural hearing loss (>95 dB) in all patients. Patients with noninherited deafness, conductive hearing loss, or syndromic forms were excluded. Written informed consents from all patients or their parents were obtained after audiological and clinical evaluations. The approval of the University of Sharjah Ethics Committee was also obtained. Saliva samples were also collected from 120 normal individuals as controls.

Mutational analysis

DNA extraction using the DNeasy Blood & Tissue Kit (Qiagen, Germany) was performed on blood samples obtained from peripheral veins. To screen the coding sequence of *GJB2* gene, 565 and 1003 bp polymerase chain reaction (PCR) products containing, respectively, exon1 and exon 2 were sequenced by the Sanger method (Sanger and Coulson, 1975). Primers were designed as follows: Ex1-F: 5′ TGG GGG CAC TTG GGG AAC TCA 3′, Ex1-R: GCA GAA ACG CCC GCT CCA GAA, Ex2-F: ACA CGT TCA AGA GGG TTT GG 3′, and Ex2-R: GGG AAA TGC TAG CGA CTG AG 3′. The amplified products were then purified using Wizard SV Gel and PCR clean-up system (Promega) and were consequently sequenced using BigDye1 Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Thermo Fisher Scientific). The resulting sequences were then purified and precipitated using ethanol/EDTA/sodium acetate precipitation method. Capillary analysis was performed in a Genetic Analyzer 3500 (Applied Biosystems, Thermo Fisher Scientific) and the data were analyzed using Sequencing Analysis software. The sequences were aligned with the published sequence of the *GJB2* gene (NM_004004.5). Heterozygous affected individuals for mutation/variation in the *GJB2* gene were analyzed for the most common deletions in the *GJB6* gene: del(GJB6-D13S1830) and del(GJB6-D13S1854), using the primers previously reported by Zaidieh *et al.* (2015).

c.-1G>A screening and pathogenicity prediction

The c.-1G>A variant, occurring in the first intron of the *GJB2* gene, abolishes a *Bcc*I restriction site. The *Bcc*I restriction pattern of the intron1–exon2 fragment (1003 bp) was used to screen 120 unrelated healthy UAE individuals. Digestion of PCR products was performed according to

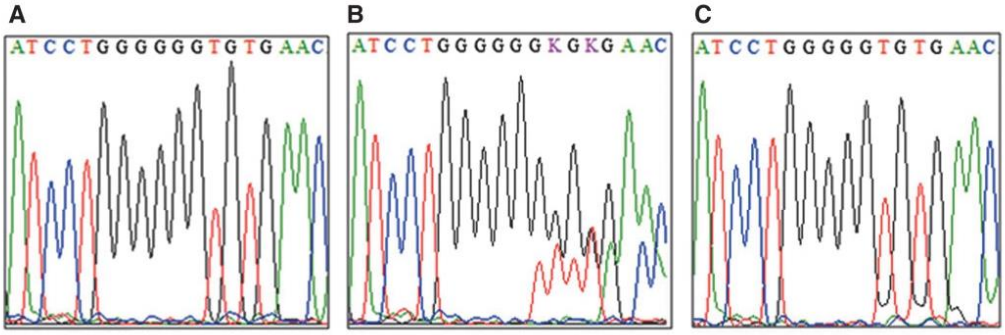

FIG. 1. The c.35delG DNA mutation: **(A)** normal homozygous individual, **(B)** carrier individual, **(C)** c.35delG homozygous individual.

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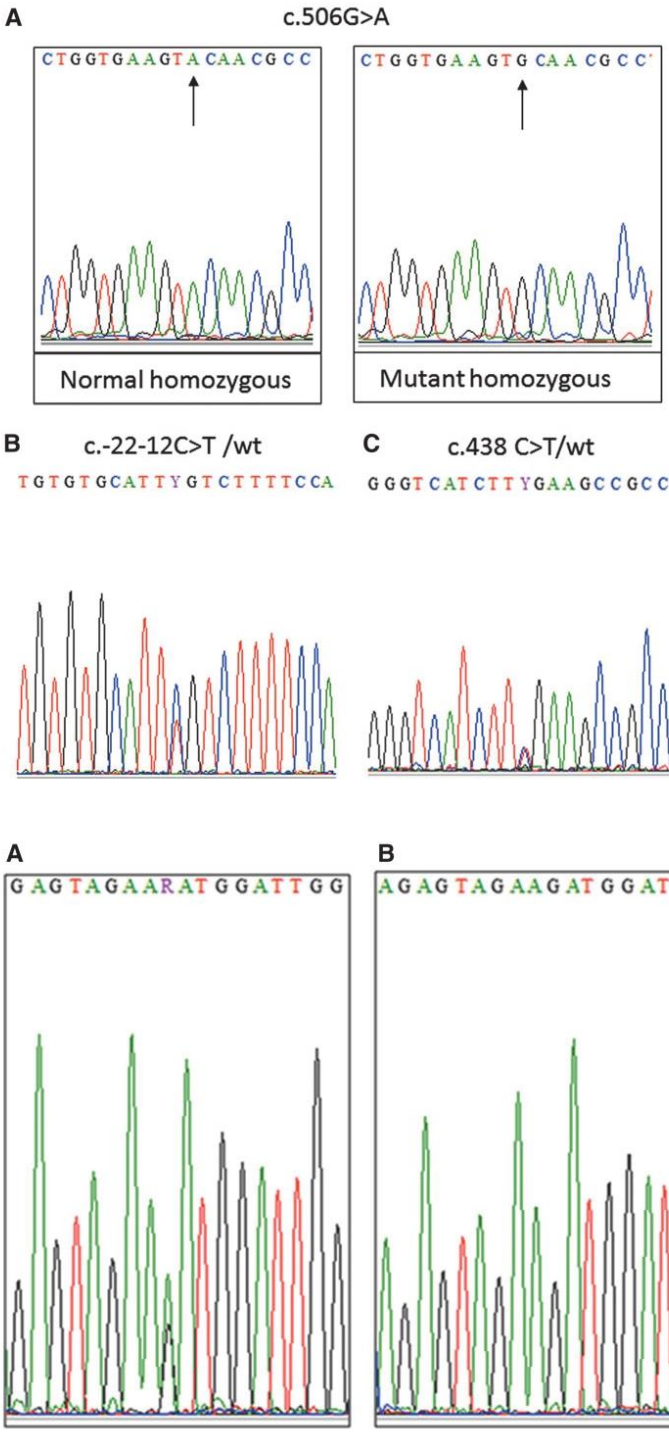

FIG. 2. Chromatograms of c.506G>A, 22-12C>T, and c.438C>T DNA variants. **(A)** c.506G>A in normal and affected individuals, **(B)** c.-22-12C>T in the heterozygous individual, **(C)** c.438C>T in the heterozygous individual. Arrows indicate the position of c.506.

FIG. 3. The DNA variation c.-1G>A: **(A)** carrier deaf individual, **(B)** normal individual.

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Table 2. Genotype Prevalence of *GJB2* Mutations in United Arab Emirates Deaf Individuals

Genotype	*No. of patients*	*Percentage of patients (%)*
c.-22-12C>T/wt	1	2
c.-1G>A/wt	1	2
c.35delG/c.35delG[a]	6	12
c.35delG/wt	2	4
c.438 C>T/wt	2	4
c.506G>A/c.506G>A[a]	3	6

[a]Genotype causative of hearing loss.

manufacturer's instructions (New England Biolabs), followed by separation on 2% agarose gels.

The sequence alignment of the *GJB2* gene was performed using the MAFFT program (www.ebi.ac.uk/Tools/msa/mafft). To predict the effect of c.-1G>A nucleotide variation, MutationTaster (www.mutationtaster.org) and NetGene2 (www.cbs.dtu.dk/services/NetGene2) were used.

Results

We analyzed 50 unrelated individuals with ARNSHL of unknown cause for *GJB2* mutations. The study of the coding region identified five different DNA variants in 15 affected individuals (30%) (Table 1).

We observed five different previously reported variants in *GJB2*. The pathogenic deletion c.35delG was detected in eight (16%) of affected individuals. Six were biallelic and two were monoallelic (Fig. 1). The second pathogenic reported variant, c.506G>A, was detected in homozygous state in three deaf individuals (6%) (Fig. 2A). In addition to these reported DNA mutations, we identified in one patient the pathogenic transition c.-1G>A in heterozygous state (2%) (Fig. 3). Moreover, two patients were carrying the silent variation c.438 C>T (p.Phe146=) in heterozygous state (4%) (Fig. 2C), and one presented the DNA variation c.-22-12C>T in heterozygous state (2%) (Fig. 2B). The mutations that were identified in this study are reported in Table 1 as allele distribution and in Table 2 as genotype distribution.

Further analysis of c.-1G>A showed that the G nucleotide at position c.-1 is conserved among several species (Fig. 4). This variation was not detected in 240 control alleles. All the programs including MutationTaster (www.mutationtaster.org) and NetGene2 (www.cbs.dtu.dk/services/NetGene2) predicted that c.-1G>A affects exon 2 splicing.

A genetic analysis of the del(GJB6-D13S1830) and del(GJB6-D13S1854) deletions in *GJB6* was accomplished in the six heterozygous individuals for DNA variation (mutation or polymorphism) in the *GJB2* gene. None of these subjects presented del(GJB6-D13S1830) and del(GJB6-D13S1854) deletions.

Discussion

Mutations in *GJB2* are highly prevalent in all nonsyndromic hereditary hearing losses, with specific mutations prevalent in distinct ethnic groups (Chan and Chang, 2014). The c.35delG mutation, within this gene, represents the most predominant variant associated with nonsyndromic autosomal-recessive hearing loss. This deletion represents a frameshift mutation that prematurely terminates the polypeptide chain of the Connexin 26 protein (p.Gly12Valfs*2). In three GCC countries, Qatar, Oman, and KSA, the frequencies of c.35delG were, respectively, 3.2%, 0%, and 6.7% (Simsek *et al.,* 2001; Al-Qahtani *et al.,* 2010; Khalifa Alkowari *et al.*, 2012). In our study, mutations in the *GJB2* gene have been related to deafness in nine (18%) patients. Eight deaf individuals had c.35delG mutation, of whom six were homozygous and two were heterozygous. In the Middle East, the c.35delG mutation is predominant in Turkey, north Iraq, and northwest of Iran; however, it is less present in Pakistan, India, Qatar, Oman, and KSA (Najmabadi and Kahrizi, 2014).

In this study, c.506G>A (p.Cys169Tyr) was found in three patients. Although this change has previously been reported as a polymorphism in the literature (Azaiez *et al.*, 2004), a recent study demonstrates that the p.Cys169Tyr mutation hinders the docking of Connexin 26 hemichannels by altering protein structure in the extracellular domain and suggests the pathogenicity of this variation as the mutated protein fails to form gap junction plaques (Zonta *et al.*, 2015).

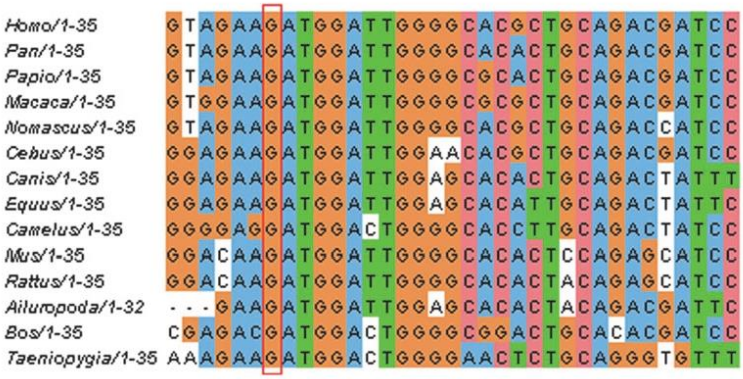

FIG. 4. Multiple sequence alignment of the *GJB2* gene region flanking nucleotide c.-1. MAFFT analysis demonstrates that Guanine-1 is well conserved in orthologs. *GJB2*, gap junction protein beta 2. Each nucleotide has a specific shade in order to differentiate between them and show consensus sequences.

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The c.438 C>T (p.Phe146Phe), found in heterozygous state in two deaf individuals, was described in several populations with low frequency and in heterozygous state (Yongyi *et al.*, 2007; Dai *et al.*, 2009; Yuan *et al.*, 2012). Although this nucleotide change does not affect the encoded amino acid, we cannot rule out the possibility that it may affect an exonic splice enhancer and cause aberrant splicing. Alternatively, modifications in DNA codon may affect the stability of the mRNA or the preference of codon usage, which, in turn, can alter the protein levels. Silent mutations have been previously shown to be disease causing both in deafness (Su *et al.*, 2007; Schultz *et al.*, 2009) and in other genetic disorders (Cartegni *et al.*, 2002).

In one patient, we found the intronic c.-22-12C>T (originally described as IVS1-12C>T) variation in heterozygous state. This nucleotide transition was first described in 2004 (Roux *et al.*, 2004). Subsequent studies confirmed the c.-22-12C>T as a normal nonpathogenic DNA variant because it was also reported in the control population (Matos *et al.*, 2011, 2013; Melo *et al.*, 2014).

Only two pathogenic mutations are known to alter splice sites in the *GJB2* gene: the transition mutation: c.-23+1G>A (originally described as IVS1+1G>A), the only mutation known to alter the donor splice site of intron 1 of the *GJB2* gene (Denoyelle *et al.*, 1999), and c.-22-2A>C (originally reported as IVS1-2A>C), which abolishes the acceptor splice sites of *GJB2* intronic region (Gandia *et al.*, 2013). In our study, variant c.-1G>A was identified in one affected individual in heterozygous state. This transition has been reported previously also in heterozygous state (Neocleous *et al.*, 2014) and it has been suggested as pathogenic mutation. The absence of c.-1G>A variant in 120 normal individuals, its conservation among different species, and its potential effect on splicing support the pathogenic effect of this transition. Hence, the functional consequence of this potential causal variant remains speculative, the establishment of *in vitro* splicing experiments and transgenic animals would be required for further analysis.

Conclusion

To the best of our knowledge, this is the first study of the prevalence of *GJB2* mutations in the UAE population. Our results indicate that c.35delG mutation, the predominant pathogenic variation of *GJB2* gene, is the most frequently seen causative mutation at a 12% ratio in affected individuals with hearing loss in our region that is followed by p.Cys169Tyr mutation. The results reported here present a preliminary step in establishing epidemiological data regarding the contribution of *GJB2* mutations in ARNSHL in the UAE population.

Acknowledgments

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Author Disclosure Statement

No competing financial interests exist.

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Clinical Exome Sequencing Identifies a Frameshift Mutation Within the *STRC* Gene in a United Arab Emirates Family with Profound Nonsyndromic Hearing Loss

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Aims: Autosomal recessive nonsyndromic hearing loss (ARNSHL) is the most common form of hereditary deafness. Despite its frequency, the diagnosis of this disorder continues to be a challenging task given its extreme genetic heterogeneity. The purpose of this study was to identify the causative mutation in a consanguineous United Arab Emirates (UAE) family with ARNSHL.
Materials and Methods: Clinical exome sequencing (CES) followed by segregation analysis via Sanger sequencing was used to identify the causative mutation. In addition, 109 deaf individuals and 50 deafness-free controls from the UAE population were screened for the identified mutation.
Results and Discussion: CES identified the *STRC* frameshift mutation c.4510del (p.Glu1504Argfs*32) as the causative mutation in this family. Moreover, segregation analysis confirmed the above finding. In addition, the absence of this variant in 109 unrelated deaf individuals and 50 healthy controls indicates that it is rare in the UAE population.
Conclusion: The present study represents the first *STRC* mutation reported in the UAE population. It also reinforces the power of next-generation sequencing in the diagnosis of heterogenous disorders such as nonsyndromic hearing loss.

Keywords: clinical exome sequencing, *STRC* gene, frameshift mutation, nonsyndromic hearing loss

Introduction

Hearing loss (HL) is one of the most frequent sensory deficits, globally affecting 1 in 1000 individuals. Most congenital cases of HL are attributable to genetic factors, and autosomal recessive nonsyndromic HL (ARNSHL) represents around 80% of nonsyndromic deafness (Morton, 1991). The diagnosis of ARNSHL is often hindered by its extreme heterogeneity with a total of 69 genes and more than 100 loci identified so far.* In addition, the mutational load of these genes can vary considerably among populations, intensifying the need for approaches that enable the efficient elucidation of ARNSHL mutations (Vona *et al.*, 2015b).

Currently, researchers are relying on next-generation sequencing (NGS)-based methodologies such as targeted sequencing panels, clinical exome sequencing (CES), or whole-exome sequencing as they enable the simultaneous detection of many genetic variations in a timely and cost-effective manner as opposed to traditional methods. These techniques have revolutionized the discovery of novel genes and mutations, particularly for diseases with distinct phenotypes such as nonsyndromic HL (Sagong *et al.*, 2016).

The *STRC* gene is an autosomal recessive HL gene that maps to DFNB16 on chromosome 15q15.3. This gene encodes a highly conserved structural protein known as stereocilin, which is essential for proper hair cell function in the inner ear (Verpy *et al.*, 2001, 2011). Recently, several studies have shown that *STRC* is an important contributor to ARNSHL, especially in those with mild to moderate HL (Francey *et al.*, 2012; Moteki *et al.*, 2016).

In this study, CES allowed the identification of a homozygous *STRC* frameshift mutation (NM_153700.2:c.4510del, p.Glu1504Argfs*32) in a consanguineous United Arab Emirates (UAE) family with ARNSHL. Screening a cohort of 109 UAE deaf individuals (familial and sporadic) and 50 deafness-free controls revealed that this mutation is rare in the UAE population. To the best of our knowledge, this is the first deafness-linked *STRC* mutation reported in the UAE.

*http://hereditaryhearingloss.org

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Materials and Methods

Study participants and clinical evaluation

A large consanguineous UAE family, including one HL patient, was recruited from the UAE Deaf Association for this study (Fig. 1A). Clinical assessment of the affected individual included medical history records, physical examination, as well as pure tone audiometry. In addition, 109 UAE deaf individuals (familial and sporadic) and 50 healthy individuals were included in this study. Saliva samples were collected from all subjects and genomic DNA was extracted from these samples using the Oragene-DNA (OG-500) Kit (DNA Genotek, CANADA) according to the manufacturer's protocol. To ensure confidentiality, only codes were used to label all DNA samples. Written informed consents were also obtained from all study participants or their parents (for subjects younger than 18 years) before sample collection. Furthermore, all experimental procedures followed in this study were approved by the Ethics Committee at the University of Sharjah (Sharjah, UAE).

CES and bioinformatic analysis

CES and standard data analysis covering a total of 6879 genes associated with known clinical phenotypes were performed for the proband (Fig. 1A). In brief, exome capture and enrichment of sheared genomic DNA followed by library construction were carried out using the SureSelect Clinical Research Exome V2 kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions. Paired-end sequencing (2×100 base pairs) with $100\times$ coverage was then conducted using Illumina HiSeq 2500/4000 system (Illumina, San Diego, CA). Only reads that were generated from high-quality sequences were aligned to the human reference genome build GRCh37/hg19 using BWA-0.7.12. Polymerase chain reaction (PCR) duplicates were then removed using Picard-1.140 and variants were called using Genome Analysis Toolkit (GATK) v2.3-9. Called variants were annotated with the in-house Variation and Mutation Annotation Toolkit 2.3.4 (VariMAT). The remaining variants were then further filtered by frequency (i.e., <0.01%) in dbSNP (www.ncbi.nlm.nih.gov/projects/SNP/), ExAC Browser (http://exac.broadinstitute.org) or gnomAD (http://gnomad.broadinstitute.org). Last, to predict the functional impact of the candidate mutation, the following bioinformatic tools were used: variant effect predictor (VEP),** Mutation Taster,† and VarSome.‡

Sanger sequencing

To ensure that the candidate mutation cosegregates with the disease phenotype, Sanger sequencing was performed using genomic DNA from individuals I-1, I-2, and II-3 (Fig. 1A). In short, PCR products corresponding to the 23rd exon of the *STRC* gene were generated using the following primers: STRC_Ex23_F (5′-CCAAAGGTTGGAAGACTCACTC-3′) and STRC_Ex23_R (5′-CCTCTCATCCAACTCTCCATTC-3′). The PCR amplicons were then treated with ExoSAP-IT PCR Product Cleanup Reagent (Applied Biosystems, Thermo Fisher Scientific) and subsequently used in the sequencing reactions conducted via the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Thermo Fisher Scientific). The resultant sequencing reactions were then purified and precipitated using the ethanol/EDTA/sodium acetate precipitation method. Afterward, capillary sequencing was carried out by Genetic Analyzer 3500 (Applied Biosystems, Thermo Fisher Scientific) and the sequences produced were analyzed using Sequencing Analysis software 6 (Applied Biosystems, Thermo Fisher Scientific) and aligned with the published sequence of the *STRC* gene (NM_153700.2).

In addition, a total of 109 deaf individuals and 50 unrelated controls from the UAE were screened for the c.4510del mutation by Sanger sequencing using the abovementioned protocol.

**http://grch37.ensembl.org/Homo_sapiens/Tools/VEP
†www.mutationtaster.org
‡https://varsome.com

Results

The consanguineous UAE family investigated in this study consisted of 12 individuals, one of whom was diagnosed with congenital bilateral profound sensorineural HL (Fig. 1B). Analysis of the family pedigree suggested a recessive mode of inheritance (Fig. 1A).

CES performed for the proband yielded a total of 375,397 DNA variations, including 5752 missense, 218 frameshift, and 75 nonsense variants. All generated variants were further filtered as follows: (1) variants described in dbSNP, ExAC Browser, or gnomAD with a frequency higher than 0.01% were excluded, (2) only homozygous variants were kept due to the family's consanguinity and recessive inheritance pattern, and (3) only variants within deafness genes were included. After prioritization based on the above criteria, only six variants remained (Table 1). Next, VEP, Mutation Taster, and VarSome were used to predict the functional impact of these remaining variants. Only one variant in the *STRC* gene (c.4510del, p.Glu1504Argfs*32) was categorized as "IMPACT=high," "disease causing," and "likely pathogenic" by VEP, Mutation Taster, and VarSome, respectively. Sanger sequencing, family segregation, and analysis of control individuals were used to rule out the implication of the remaining five variants.

To validate the above findings, Sanger sequencing of the 23rd exon of the *STRC* gene was carried out for the proband, the parents, one normal hearing sibling (II-3, I-1, I-2, II-1), 109 deaf individuals, and 50 deafness-free controls. Our results confirmed the homozygous mutant genotype of the proband and showed that both parents were heterozygous for the *STRC* variant (Fig. 1C, D), while the healthy sibling was homozygous for the wild-type allele (Fig. 1E). These findings indicate that the c.4510del variant cosegregates with HL in this family. The absence of this variant in 109 unrelated deaf individuals and 50 healthy controls indicates that it is rare in the UAE population. Collectively, these results indicate that the c.4510del variant in the *STRC* gene is the mutation causing ARNSHL in this family.

Discussion

In the present study, CES was used to identify the causative mutation in a consanguineous UAE family with ARNSHL. First, Sanger sequencing of the proband confirmed the absence of mutations in the *GJB2* gene, the most common ARNSHL gene in the UAE population (Tlili *et al.*, 2017). Afterward, CES was performed for the proband, and via

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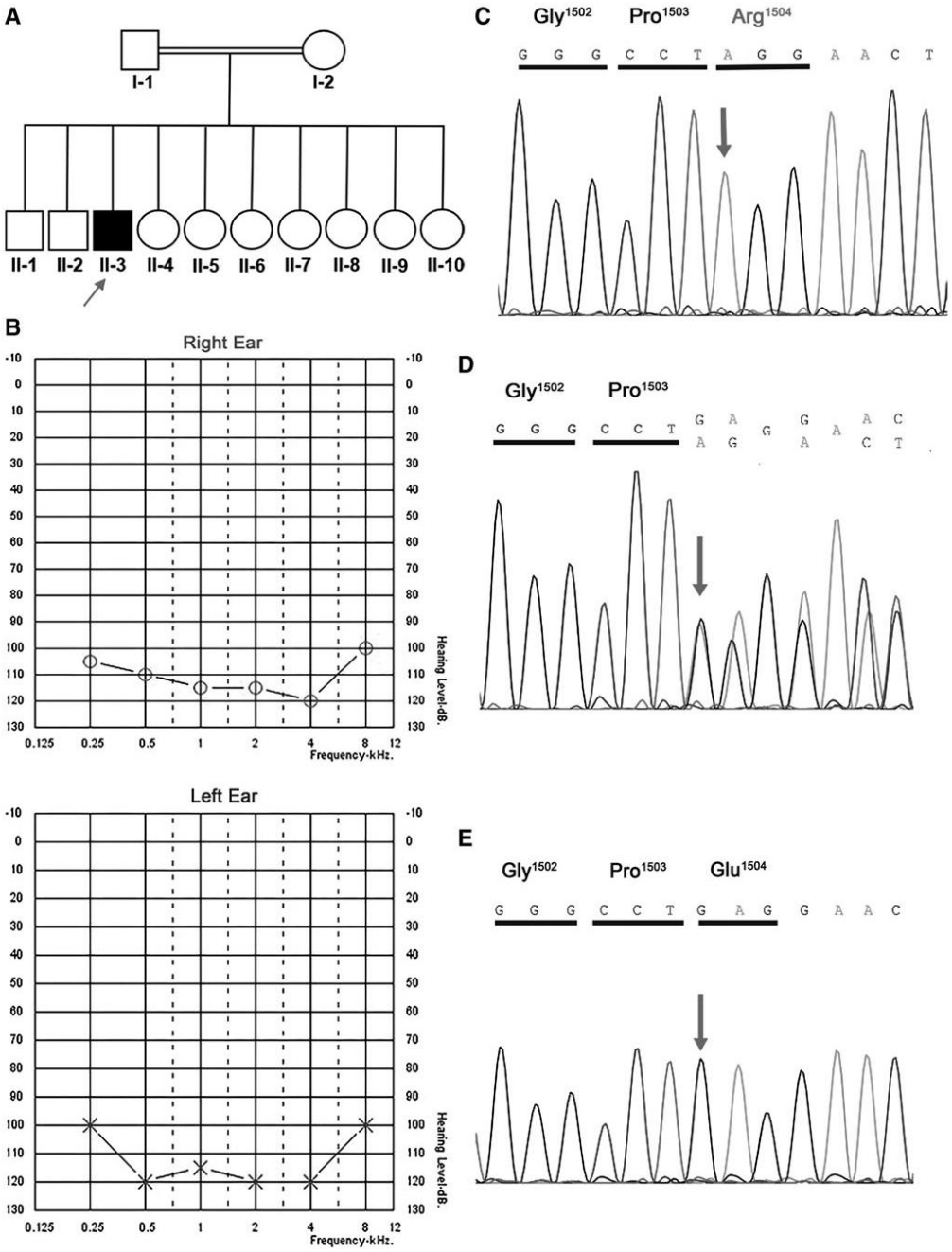

FIG. 1. Pedigree, Audiograms, and Electropherograms. **(A)** Pedigree of the affected family. *Arrow* denotes the proband. **(B)** Recent audiograms of individual II-3 (thresholds measured at the age of 30), exhibiting bilateral profound sensorineural hearing loss. **(C)** Electropherogram of a homozygous mutant individual with the c.4510del frameshift mutation in the *STRC* gene. **(D)** Electropherogram of a heterozygous individual. **(E)** Electropherogram of a homozygous wild-type individual. **(C-E)** *Arrows* indicate the mutation's position.

Table 1. Remaining Homozygous Deafness Gene Variants After Filtration of Clinical Exome Sequencing Results

Gene	*DNA change*	*RS-ID*	*Class*
OTOF	NM_004802.3:c.2676+266_2677-177del	NA	Intronic
OTOGL	NM_173591.3:c.6241−56_6241-43dup	NA	Intronic
OTOGL	NM_173591.3:c.6709−112G>A	rs200447966	Intronic
STRC	NM_153700.2:c.4510del	rs759816064	Frameshift-del
WBP2	NM_012478.3:c.59+208T>G	rs532139755	Intronic
COL9A3	NM_001853.3:c.1287+84A>C	rs8119815	Intronic

NA, not available.

Sanger sequencing, a homozygous frameshift mutation in the *STRC* gene was identified as the causative mutation in this HL family.

STRC was first reported as an ARNSHL gene by Verpy *et al.* (2001) on the analysis of two families originating from Pakistan and France. Since then, *STRC* mutations have been identified in many populations, including the United States (Francey *et al.*, 2012), Turkey (Bademci *et al.*, 2014), Germany (Vona *et al.*, 2015a), Korea (Sagong *et al.*, 2016), Japan (Moteki *et al.*, 2016), Belgium (Sommen *et al.*, 2016), the Dutch (Zazo Seco *et al.*, 2017), and Czech Republic (Plevova *et al.*, 2017). In fact, in both the United States (particularly in Caucasians and Hispanics) (Sloan-Heggen *et al.*, 2016) and Czech Republic, *STRC* mutations are the second-most frequent cause of hereditary HL after *GJB2*. The majority of described *STRC* mutations have been in the form of copy number variations mostly leading to the complete deletion of the gene, however, point mutations, small deletions or insertions, and gene conversions have also been reported (Markova *et al.*, 2018). The growing evidence linking *STRC* mutations to nonsyndromic HL reflects its significant role in this otherwise genetically heterogeneous disease.

The *STRC* gene is located on chromosome 15q15.3 and comprises 29 exons. Stereocilin, a protein consisting of 1775 amino acids, is encoded by *STRC* and is often defective in DFNB16. The first 22 amino acids of this protein are predicted to code for a signal peptide, which on cleavage would leave a mature 193 kDa protein (Verpy *et al.*, 2001). Stereocilin is expressed in the stereocilia of the hair cells found within the inner ear. Inside the inner ear, two types of hair cells exist: outer hair cells (OHCs), which act as cochlear amplifiers, and inner hair cells, which have a sensory role, transmitting information to the brain. In the OHCs, the tips of the tallest stereocilia are embedded in the tectorial membrane, and stereocilia are coupled together by horizontal top connectors. Stereocilin is a component of both the horizontal top connectors and tectorial membrane attachment links. In fact, in stereocilin null mutant mice ($Strc^{-/-}$), the horizontal top connectors are absent, resulting in an imprecise alignment among stereocilia, in turn leading to a disorganized hair bundle and progressive deafness in these mice (Verpy *et al.*, 2008). This is similar to the progressive nature of several reported cases of *STRC*-linked HL (Knijnenburg *et al.*, 2009; Moteki *et al.*, 2016).

The UAE family reported here had congenital profound HL. Although many reported cases of *STRC*-instigated deafness showed mild to moderate phenotypes, severe to profound phenotypes have also been reported (Francey *et al.*, 2012). The *STRC* mutation c.4510del was previously suggested to be a causative mutation for ARNSHL in a western European patient, where it was reported in a heterozygous state along with a hemizygous deletion (Sommen *et al.*, 2016). In contrast, our study identified this mutation in a homozygous state through segregation analysis. Moreover, the absence of c.4510del in 109 unrelated deaf individuals and 50 healthy controls from the UAE indicates that it is rare in this population, which is comparable with its low frequency (~0.00004) in both ExAC Browser (5/121342) and gnomAD (9/246170). In addition, according to the new American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) guidelines adapted by the HL Variant Curation Expert Panel, this mutation can be classified as PVS1_Strong, PM2 and PP1_Moderate giving it a final classification of ''Pathogenic'' (Oza *et al.*, 2018).

The detection of *STRC* mutations via NGS technologies is often complicated by the high homology between *STRC* and its neighboring *pSTRC* pseudogene (99.6% of the coding sequence and 98.9% including intronic regions), the latter of which is rendered inactive by a nonsense mutation in the 20th exon (Verpy *et al.*, 2001; Francey *et al.*, 2012). This complexity arises due to the fact that homologous sequences can cause the misalignment of sequence reads leading to false positive and negative variant calls (Sipos *et al.*, 2012). However, this impediment is evaded in the case of the *STRC* mutation c.4510del as it is within the 23rd exon, which is one of the very few exons that align cleanly to the *STRC* reference sequence.

The deleterious mutation c.4510del causes an amino acid change (p.Glu1504Argfs*32) that, if translated, would omit the last 272 amino acids of stereocilin in the longest two isoforms (ENST00000450892.2 and ENST00000541030.1) of the *STRC* gene. Moreover, by using bioinformatic tools, we found that this mutation is located in the intronic regions of the rest of the isoforms and is predicted to have a deleterious effect at the mRNA level (intron retention or nonsense mediated decay). In addition, on the review of reported *STRC* deafness mutations (http://deafnessvariationdatabase.org/), we found that all of them are located within the longest isoform (ENST00000450892.2), leading us to hypothesize that this isoform is the one responsible for the hearing mechanism.

Conclusion

In conclusion, CES allowed the efficient identification of the homozygous c.4510del frameshift mutation as the causative mutation in a consanguineous UAE family with

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ARNSHL. These results add the *STRC* gene to the spectrum of nonsyndromic HL genes in the UAE population. They also emphasize the importance of using NGS-based techniques in genetic testing, especially in populations with high consanguinity rates.

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Author Disclosure Statement

No competing financial interests exist.

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RESEARCH ARTICLE

Identification of a novel frameshift mutation in the *ILDR1* gene in a UAE family, mutations review and phenotype genotype correlation

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Abstract

Autosomal recessive non-syndromic hearing loss is one of the most common monogenic diseases. It is characterized by high allelic and locus heterogeneities that make a precise diagnosis difficult. In this study, whole-exome sequencing was performed for an affected patient allowing us to identify a new frameshift mutation (c.804delG) in the Immunoglobulin-Like Domain containing Receptor-1 (*ILDR1)* gene. Direct Sanger sequencing and segregation analysis were performed for the family pedigree. The mutation was homozygous in all affected siblings but heterozygous in the normal consanguineous parents. The present study reports a first *ILDR1* gene mutation in the UAE population and confirms that the whole-exome sequencing approach is a robust tool for the diagnosis of monogenic diseases with high levels of allelic and locus heterogeneity. In addition, by reviewing all reported *ILDR1* mutations, we attempt to establish a genotype phenotype correlation to explain the phenotypic variability observed at low frequencies.

Introduction

Deafness is one of the most common sensorineural disorders, affecting one in 1000 individuals. Most congenital cases of deafness have a genetic etiology, and nonsyndromic hearing loss (NSHL) accounts for approximately 80% of genetic deafness [1]. To date, a total of more than 100 NSHL genes have been identified (http://hereditaryhearingloss.org), and most mutations in these genes are inherited in an autosomal recessive pattern. Both allelic and genetic heterogeneities of NSHL make conventional methods (e.g., Sanger sequencing) expensive and time consuming [2]. The development of an efficient and cost-effective approach, whole-exome sequencing (WES), has successfully helped researchers identify new mutations and genes responsible for NSHL [2–7].

The immunoglobulin-like domain containing receptor 1, a predicted type 1 transmembrane protein with a crucial role in the epithelial barrier function in the ear, is encoded by the *ILDR1* gene [8,9]. In 2011, Borck et al., [10] reported eight different homozygous *ILDR1*

mutations in affected individuals from 11 unrelated families. Further studies confirmed the implication of this gene in NSHL [2,11,12].

In the present study, we identify a new homozygous frameshift mutation in *ILDR1* in a UAE consanguineous family with autosomal recessive non-syndromic hearing loss (ARNSHL). The mutation p.Glu269ArgfsTer4 in this family is caused by c.804delG. Further, we screened a cohort of 50 UAE familial and sporadic individuals with hearing loss and found that this mutation is unique to this family. To the best of our knowledge, this is the first *ILDR1* identified mutation causing hearing loss in a deaf family from UAE.

Materials and methods

Patients

In this study, we investigated a UAE consanguineous family transmitting an autosomal recessive non-syndromic severe to profound sensorineural hearing loss (Fig 1). Informed consent was obtained from all participants and from parents of subjects younger than 18 years of age. Clinical history interviews and physical examinations of the family members ruled out the implication of environmental factors for causing the NSHL. Saliva samples were obtained from 12 family members, including 5 hearing-impaired individuals. Saliva samples were also collected from 50 unrelated patients (26 sporadic and 24 familial cases) who were admitted to the UAE deaf association as well as 120 normal individuals as controls. The privacy and anonymity of all participants were protected and only codes were used to label DNA samples. Genomic DNA was extracted from saliva samples using Oragene-DNA (OG-500) Kit (DNA Genotek, CANADA) dx.doi.org/10.17504/protocols.io.jhtcj6n. Written informed consents from all patients or their parents were obtained following audiological and clinical evaluations. The experimental procedures were approved by the Ethics Committee from the University of Sharjah (Sharjah, UAE).

Whole-exome sequencing and bioinformatics analysis

Sequencing library construction, exome capture, sequencing, and standard data analyses for the affected children in this family was performed by Sengenics. Exome capturing and

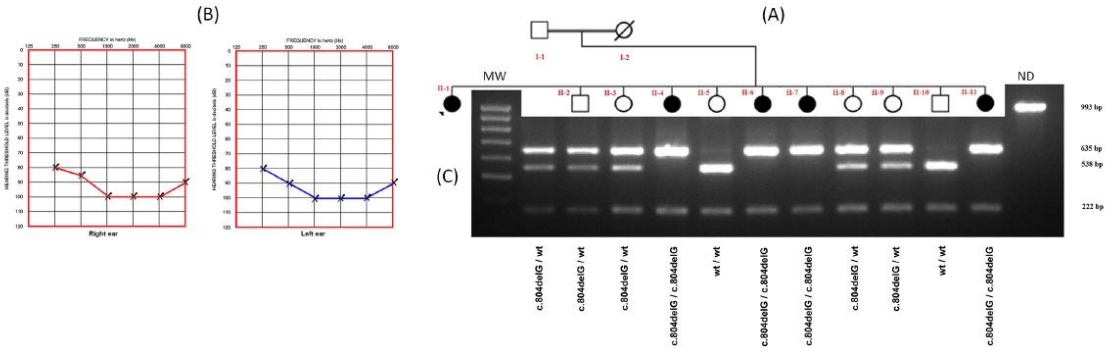

Fig 1. Pedigree of the affected family, Audiogram and PCR-RFLP analysis. (A) Pedigree of the affected family with nonsyndromic hearing loss. Arrow denotes the proband. (B) Audiogram of the proband individual II-1 exhibiting bilateral, severe to profound sensorineural hearing loss. (C) Results of PCR-RFLP analysis of DNA of the affected family with nonsyndromic hearing loss. A 993 bp PCR fragment is digested with FauI restriction enzyme. The wildtype DNA is cleaved into four fragments 538, 222, 136 and 97 bp, whereas the c.804delG mutant allele is cleaved into three fragments 635, 222 and 136 bp in length. MW: DNA Ladder (100bp DNA Ladder, REF G2101) (Promega, USA); ND: undigested PCR product.

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enrichment was carried out using SureSelect All Exon V5 kit (Agilent Technologies, Santa Clara, CA, USA) following the manufacturers' protocols. Whole exome sequencing was carried out on Illumina HiSeq 2500 system (Illumina, San Diego, CA, USA). Paired end (2×100 bases) DNA sequence reads that passed the quality control i.e phred score > 20 were mapped to the human reference genome build hg19/GRCh37 using the BWA [13] and SAM tools [14] was used for processing BAM files. Genome analysis tool kit (GATK) v2.7.2) [15] was used for calling variants from BAM files. Variants were annotated with gene, existing variations, consequences from dbSNP (build 137), SIFT v5.0.2 [16] and polyphen v2.2.2 [17] using Ensembl Variant Effect Predictor v73 (VEP) [18]. Known variants were annotated by dbSNP and unannotated variants with serious predicted consequences were identified based on SIFT and polyphen which were considered as novel variants. Variants were filtered for increased accuracy using following steps: a) variants were filtered at the read depth (DP) > = 10 b) Variants with >10% i.e > 0.1 minor allele frequency based on 1000 Genome project ((http://www.1000genomes.org/data) dx.doi.org/10.17504/protocols.io.jhscj6e.

Sanger sequencing

Sanger sequencing was performed on available samples from all affected family members to determine whether the potential mutation in the causative gene co-segregated with the disease phenotype. In order to amplify exon 7 of the *ILDR1* gene, we designed the following primers: ILDR1-7F: `TTGATGTCCTGATTCTGAGG` and ILDR1-7R: `CTCTGTGGTGGAATGAGAGG`. The amplified products were then purified using Wizard SV Gel and PCR Clean-up system (Promega, USA) and were consequently sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Thermo Fisher Scientific, USA). The resulting sequencing reactions were then purified and precipitated using Ethanol/EDTA/Sodium acetate precipitation method. Capillary sequencing was performed in a Genetic Analyzer 3500 (Applied Biosystems, Thermo Fisher Scientific, USA) and the data were analyzed using Sequencing Analysis software. The sequences were aligned with the published sequence of the *ILDR1* gene (NM_001199799.1) dx.doi.org/10.17504/protocols.io.jhvcj66.

c.804delG mutation screening

The novel c.804delG mutation, occurring in the seventh exon of the *ILDR1* gene, abolishes a FauI restriction site. The FauI restriction pattern of the exon7 fragment (1003 bp) was used to screen 50 deaf individuals and 120 unrelated healthy UAE individuals. Digestion of PCR products was performed according to manufacturer's instructions (New England Biolabs, USA), followed by separation on 2% agarose gels dx.doi.org/10.17504/protocols.io.jhwcj7e.

In silico analysis of DNA variants

In order to determine the consequence of *ILDR1* reported mutations on splicing and translation initiation, we used Human Splicing Finder (version 3.0) (http://www.umd.be/HSF3/) and ORF finder (http://www.geneinfinity.org/sms/sms_orffinder.html) respectively dx.doi.org/10.17504/protocols.io.jhxcj7n.

Results

Molecular analysis

A large UAE consanguineous family including five patients with congenital deafness, was investigated (Fig 1A). Clinical examination of all affected individuals ruled out any association with other symptoms and showed no conductive abnormality S1 Table. Hence, we could

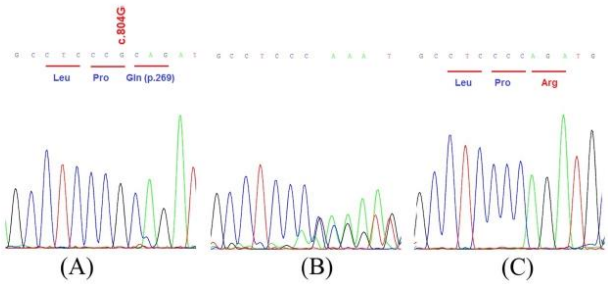

Fig 2. Electropherograms. Homozygous normal individual (A), Heterozygous individual (B), and affected individual with the c.804delG pathogenic variant in the *ILDR1* gene (NM_001199799.1) (C).

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qualify the hearing loss in the corresponding family as a nonsyndromic autosomal recessive hearing loss. As a routine screening in our laboratory, we first sequenced the *GJB2* gene (Tlili et al., 2017, under revision), the most common gene in ARNSHL, in affected individuals and found no mutations (data not shown). Thus, we performed a WES analysis for the DNA of individual II-1 DNA (Fig 1A). A total of 123484 DNA variations were identified with 6393 missense, 186 frameshift and 64 nonsense variants. This data has been further filtered as follows: (i) only homozygous variants were considered as the disease is recessive and the family was consanguineous, (ii) common variants found in the UAE population and available in our internal database were removed, (iii) variants described in dbSNP (https://www.ncbi.nlm.nih.gov/projects/SNP/) or Exac Browser (http://exac.broadinstitute.org/) with a frequency higher than 0.05% were excluded. After this filtering, only 70 non described DNA variations were kept, 67 among them were in noncoding regions, one silent variant in *HEG1* gene (c.51C>T, p.Leu17Leu), one insertional variation c.308_309insCTG (p.Ala103_Val104insTrp) in the *HLA-DRB5* and one nucleotide deletion c.804delG (p.Glu269ArgfsTer4) in the *ILDR1* gene. These three variants have been analyzed by Mutation Taster algorithm in order to predict their functional impact. Using this analysis, we found that *HEG1* and *HLA-DRB5* variations are predicted to be polymorphisms and only the *ILDR1* variation is predicted to be a "disease causing" variant. To validate this finding and the WES results, we sequenced exon 7 of the *ILDR1* gene in the proband individual and her family members (Fig 2). Our analysis revealed the cosegregation of the c.804delG variant with the hearing loss observed in our studied family. This cosegregation has been also confirmed by PCR-RFLP using FauI restriction enzyme (Fig 1C). In fact, all affected individuals were homozygous for this deletion, both parents were heterozygous and unaffected siblings were either heterozygous or normal homozygous for the wildtype allele. Furthermore, using this restriction enzyme, we demonstrated that this DNA variation was not present in 50 unrelated deaf individuals and 120 normal hearing UAE control individuals. Together, all these analyses and results suggest that the c.804delG deletion within the *ILDR1* gene represents the pathogenic mutation responsible for the ARNSHL observed in this UAE family.

In silico analysis

In order to establish a genotype phenotype correlation, we reviewed all reported mutations within the *ILDR1* gene and performed an insilico analysis. The study of c.3G>A (p.Met1?) by ORF finder (Table 1, Fig 3) revealed that in the presence of A at position c.3, the next potential initiation codon that can be used is located at position 136 downstream the original one

Table 1. *ILDR1* reported mutations, described effects and predicted consequences using insilico analysis.

DNA Mutation	Described protein mutation	Reference	Predicted insilico protein mutation (this study)
c.3G>A	Use of the next downstream ATG out-of-frame, potentially producing a 43 amino acid polypeptide with no similarity to ILDR1.	[10]	p.Met1Ile*ext*+136
c.59-5_88del	N/D	[10]	p.Gly20_Thr31del
c.82delG	p.V28SfsX31	[3]	-
c.206C>A	p.Pro69His	[19]	-
c.290 G>A	p.Arg97Gln	[10]	-
c.305T>A	p.Val102Glu	[12]	-
c.325_333dupAATGAGCCC	p.Asn109_Pro111dup	[11]	-
c.411delG	p.Trp137CysfsX25	[10]	-
c.499+1G>A	N/D	[10]	p.Trp168LysfsTer47
c.583C>T	p.Gln195X	[10]	-
c.804del G	p.Glu269ArgfsTer4	Present study	-
c.820C>T	p.Q274X	[2]	-
c.942C>A	p.C314X	[3]	-
c.1032delG	p.Thr345ProfsX20	[10]	-
c.1135G>T	p.Glu379X	[10]	-
c.1180delG	p.Glu394SerfsX15	[10]	-
c.1217-1218delTC	p.S406X	[12]	-
c.1358G>A	p.Arg453Gln	[10]	-

N/D: not determined

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(p.Met136). This alternative translation produces an *ILDR1* protein lacking the signal peptide (SP) and 78% of the Ig domain, however the remaining regions are identical to the wildtype form as the use of p.Met136 doesn't affect the normal frame. The analysis of the c.59-5_88del mutation using HSF (Table 1, Fig 3), revealed a new potential splice acceptor site "cttggcacacagAA" in frame with a consensus value of 84.34 (the value of the original one "tcttggcttttagGG" abolished with this mutation, is 84.05). Taking into consideration this new acceptor site, the predicted consequence of c.59-5_88 del mutation on the protein will be an in-frame

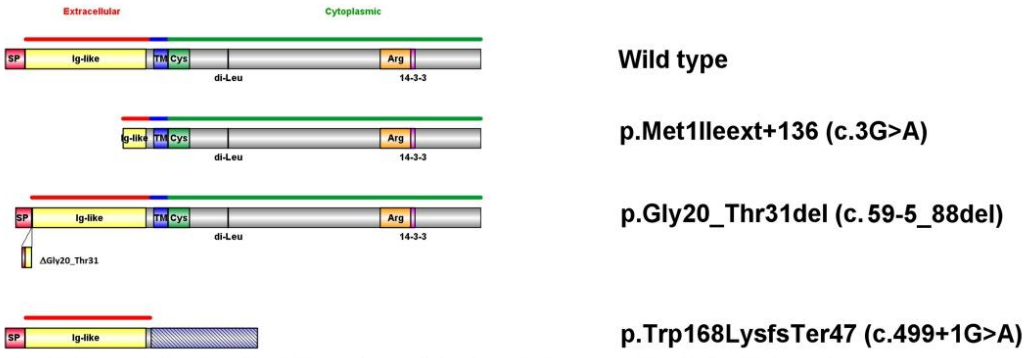

Fig 3. In silico analysis and predicted effect at the protein level of c.3G>A, c.59-5_88del and c.499+1G>A mutations.

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deletion of 4 aa of the SP and 8 aa of the Ig domain (p.Gly20_Thr31del). Concerning the intronic variation c.499+1G>A, our HSF analysis revealed that the wildtype donor site "TACgta agt" with a consensus value of 87.01 has been lost and probably the totality of intron 4 (290 bp) will be included in the coding region. This will probably produce a mutant *ILDR1* protein (p.Trp168LysfsTer47) of 214 aa only (Table 1, Fig 3). For the remaining 14 reported mutations, none of them creates a new potential initiation codon, however a few only create new splice sites but their score values were less than the normal ones (data not shown).

Discussion

We report a UAE consanguineous family affected with ARNSHL. The affected members have severe to profound congenital hearing loss. Further clinical examinations ruled out the association with any other symptom, and suggested a nonsyndromic form of hearing loss in the studied family. We first analyzed the affected individual II-1 for mutations in the *GJB2* gene, the most common gene in ARNSHL in UAE population (Tlili et al., 2017, under revision) and found no mutations. As next-generation sequencing-based methods (WES and specially the gene panel test) became a preferable strategies to screen mutations in several genes at the same time, taking into consideration their cost effectiveness and reduced time consumption as compared to other genetic testing methods [2], we performed WES analysis on the proband and we were able by Sanger sequencing to identify the causative mutation in the *ILDR1* gene. In 2011, this gene has been implicated in ARNSHL in 11 families linked to *DFNB42* locus [10]. So far, several mutations in the *ILDR1* gene have been described [2,3,11,12,19,20].

The longest isoform of the *ILDR1* gene produces a transmembrane protein with 546 amino acids. It contains a signal peptide, an extracellular immunoglobulin (Ig) superfamily domain, a transmembrane domain, a cysteine-rich and an arginine-rich domain, an LSR (lipolysis stimulated lipoprotein receptor) domain, a dileucine motif, and a 14-3-3 binding site [8,10]. The

Table 2. Mutations in *ILDR1* gene and associated phenotypes.

Mutation (cDNA)	Mutation (protein)	Affected Domain (s)	Human phenotype
c.3G>A	p.Met1Ile*ext*+136	Signal peptide and extracellular domain	Moderate to profound
c.59-5_88del	p.Gly20_Thr31del	Signal peptide and extracellular domain	Moderate to profound
c.82delG	p.V28SfsX31	Extracellular, transmembrane and intracellular domains	N/A
c.206C>A	p.Pro69His	Extracellular domain	Post-lingual onset and partial deafness
c.290 G>A	p.Arg97Gln	Extracellular domain	N/A
c.305T>A	p.Val102Glu	Extracellular domain	Severe to profound
c.325_333dupAATGAGCCC	p.Asn109_Pro111dup	Extracellular domain	Moderate to profound
c.411delG	p.Trp137CysfsX25	Extracellular domain	N/A
c.499+1G>A	p.Trp168LysfsTer47	Transmembrane and intracellular domains	Severe
c.583C>T	p.Gln195X	Intracellular domain	Severe to profound
c.804del G	p.Glu269ArgfsTer4	Intracellular domain	Severe to profound
c.820C>T	p.Q274X	Intracellular domain	N/A
c.942C>A	p.C314X	Intracellular domain	N/A
c.1032delG	p.Thr345ProfsX20	Intracellular domain	Severe
c.1135G>T	p.Glu379X	Intracellular domain	Severe to profound
c.1180delG	p.Glu394SerfsX15	Intracellular domain	Severe
c.1217-1218delTC	p.S406X	Intracellular domain	Moderate to profound
c.1358G>A	p.Arg453Gln	Intracellular domain	Severe to profound

N/A: not available

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c.804delG (p.Glu269ArgfsTer4) mutation is predicted to produce a mutant protein lacking the arginine-rich domain and the 14-3-3 binding site. These two regions are very conserved among several species, which suggests an important biological function. In fact, arginine-rich motifs are found in important regulatory complexes, and they are predicted to mediate protein-protein interaction [21] or to bind a functional domain of a protein to an RNA [22,23]. The 14-3-3 binding sites are also important since 14-3-3 domain bind target proteins and modulate their activity, stability and subcellular localization. Furthermore, 14-3-3 domain contributes to protein complex formation [24]. In 2015, Higashi et al., documented that hairs cells in the *Ildr1* knockout mice develop normally, but begin to degenerate two weeks after birth. Thus, they suggest that in the absence of ILDR1 gene, hair cells undergo a postnatal degeneration. At P35, all knockout mice had profound sensorineural hearing loss associated with a complete loss of outer hair cells and a disorganization of most stereocilia in inner hair cells [25].

The phenotype of the UAE family reported here, is similar to some of the previously reported DFNB42 families. Affected members have severe to profound sensorineural hearing loss. A review of all mutations reported up to date, the in-silico analysis of their consequences at the protein level (Table 1 and Fig 3) and the correlation with their associated phenotype (Table 2), suggested that the mutation in the extracellular domain, induces moderate deafness that is detected at low frequencies, but any mutation disturbing the intracellular domain of the *ILDR1* protein will result in a severe deafness at low frequencies. An exception to our finding are p.Val102Glu and p.S406X mutations described by Mehrjoo et al., 2015 (Table 2). Hence, we suggest for DFNB 42 linked families with moderate hearing loss at low frequencies to screen exons 1, 2 and 3 as they encode the extracellular domain of *ILDR1* gene.

As low frequencies reflect the activity of the apical part of the cochlea, we suggest that the extracellular domain of *ILDR1* protein is not very crucial in the apical part of the organ of Corti. However, any alteration of the intracellular domain affects the totality of this organ and results in a severe to profound hearing loss phenotype. This hypothesis is supported by *ILDR1* knockout mice results. [25–27]. In fact, all knockout mice unable to express ILDR1 intracellular domain showed severe to profound deafness. Furthermore, scanning electronic microscopy and/or immunocytochemistry analysis in adult mutants, revealed that all outer and inner hear cells were degenerated in the apical, middle and basal turns of the cochlea [25–27]. Moreover, differential gene expression profiles along the axis of the mouse cochlea have been established [28,29]. As an example, the *Tectb* gene, responsible for low frequency hearing loss in mouse [30], is 23-fold more expressed in the apical turn, which is sensitive to low frequencies, compared to middle and basal turns [29]. To explain the phenotypic variability related to *ILDR1* mutations, we suggest a mechanism in which a differential protein functional profile along the axis of the cochlea occurs; in the middle and basal turns of the cochlea, both extracellular and cytoplasmic domains are essential for the structural integrity and functionality of inner ear tricellular tight junctions, however in the apical turn, only the cytoplasmic domain is required.

In addition to the previous phenotype-genotype correlation based on the position of the mutation, we can also consider the nature of the mutation as a determinant factor for the phenotypic variability of ILDR1 mutants. In fact, most of mutations associated with severe to profound hearing loss (except p.Val102Glu and p.Arg453Gln) are nonsense mutations that likely lead to nonsense mediated decay of the mutated mRNA and prevent translation of ILDR1 protein. On the other hand, non-truncated mutations associated with moderate to profound hearing defect (except p.S406X) seem more often to allow production of the mutated protein. Based on these observations, we can suggest that the presence of the mutated ILDR1 protein in the cochlea results in a moderate to profound hearing loss. However, the absence of the ILDR1 protein results in a severe to profound phenotype. This gradient of hearing impairment related

to inactivating variants (stop mutations or frame shifts) and non-inactivating variants (missense mutations) has been reported in several studies. As example, Cryns et al, found in 2004 that 35delG homozygotes (an inactivating mutation) have significantly more hearing impairment, compared with 35delG/non-35delG compound heterozygotes. They also showed that people with two non-35delG mutations have even less hearing impairment [31].

Conclusion

To conclude, this report provides a review of all *ILDR1* mutations with phenotype genotype correlation alongside the molecular diagnosis of a consanguineous UAE family with five deaf individuals found to be homozygous for a novel frameshift mutation c.804delG.

Supporting information

S1 Table. Clinical assessment of affected individuals.
(DOCX)

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