Genetic study of autosomal recessive nonsyndromic sensorineural hearing loss in Kuwaiti children

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Background
The prevalence of congenital hearing loss was found to be one in 1000 of live births. Fifty percent of the cause is genetic and autosomal recessive nonsyndromic sensorineural hearing loss (ARNSNHL) is responsible for 80% of the genetic causes.

Study design
Descriptive cross-sectional study.

Objectives
To study the genetic causes of ARNSNHL, mainly mutation in the gene encoding *connexin26 (Cx26)*, and to correlate the identified gene and mutation with the degree and configuration of hearing loss, the progressiveness of hearing loss, as well as its relation to language development.

Patients and methods
One hundred children, age ranging from 6 months to 18 years, presenting with congenital ARNSNHL were chosen. Behavioral observation audiometry or pure tone audiometry to identify the hearing threshold level of the children was performed. Imittancemetry, otoacoustic emissions, auditory brainstem response, and computed tomography scan study were also undertaken. In addition, genetic tests to detect *Cx26* mutations using a PCR and primers, as well as sequencing using different primers were also undertaken.

Results
Out of the total of 100 cases, 15 children were shown to have positive results for *Cx26*; nine of these were heterozygous and six were homozygous. Twelve participants (80%) among the *Cx26* cases were due to 35delG. Out of the nine children who were heterozygous, six showed positive results for D1 (35delG), whereas three children were found to have positive results for D2 (G2A at location base 71). All the six homozygous cases were shown to be positive for D1 (35delG). Thus, the results revealed that 80% of the positive genetic results cases had 35delG.

Conclusion
Eighty percent of the cause of ARNSNHL in a Kuwaiti population was the 35delG mutation and 20% was due to G2A at location base 71. Of all the cases, 57% showed a positive family history of hearing loss. The homozygous cases presented with more severe clinical pictures compared with the heterozygous cases.

Keywords:
35delG, auditory brainstem response, ARNSNHL, *connexin26*, genetic hearing loss, pure tone audiometry, transient evoked otoacoustic emission

Introduction

Hearing loss is the most common sensory deficit in humans. Roughly, one child in a 1000 is born with hearing impairment significant enough to compromise the development of normal language skills. Hearing loss can be caused by environmental factors as well as genetic factors. It is estimated that 50–75% of all childhood deafness is due to hereditary causes [1].

There are two main forms of genetic hearing loss: syndromic and nonsyndromic. Children with syndromic hearing loss have other clinical features in addition to

hearing loss. About 15–30% of the hereditary hearing loss is syndromic, whereas the vast majority (70%) is nonsyndromic [2].

Angeli *et al.* [1] reported that four connexins have been implicated: *Cx26* (GJB2), *Cx31* (GJB3), *Cx30* (GJB6), and *Cx43* (GJAI). GJB2 is the first nonsyndromic sensorineural deafness gene to be identified. It is estimated that mutations in GJB2 account for 50% of recessive nonsyndromic hearing loss; GJB2 encodes *Cx26* [3].

It is clear that the GJB2 gene mutations are a leading cause of deafness in nearly all populations that have been

studied. This gene mutation has not been thoroughly investigated in Kuwaiti hearing impaired children. Thus, in collaboration with the Audiology department, Faculty of Medicine, Cairo University, the department of Genetics in Kuwait University addressed this issue, hoping to identify the prognostic variables that could guide the rehabilitation program for these patients.

The aim of this work is to study the genetic causes of autosomal recessive nonsyndromic sensorineural hearing loss (ARNSNHL), mainly mutation in the gene encoding *Cx26*, and to correlate the identified gene and mutation with the degree and configuration of the hearing loss, the progressiveness of hearing loss, as well as its relation to language development in Kuwaiti children.

Patients and methods

One hundred Kuwaiti children attending the Audiology Outpatient Department (OPD) in the Sheikh Salem Al-Ali center for Audiology and Speech, Kuwait University, in collaboration with the Audiology Department, Faculty of Medicine, Cairo University, in the period between October 2007 and June 2009 participated in this study. They had been referred from different clinics in Kuwait, mainly ENT, Pediatrics, and Genetics. The children who were chosen for this study were those who had congenital, ARNSNHL. Children who showed any other causes of sensorineural hearing loss (SNHL), for example, prenatal, perinatal, and postnatal problems, otitis media, meningitis, etc. were excluded. There was a large variation in the age group among those who attended the Audiology OPD; the minimum was 6 months, whereas the maximum was 18 years, with a mean age of 7.43 ± 5.73 years. Written informed consents were obtained from the parents before enrollment in this study. The study protocol was approved by the Otolaryngology Department Council of Cairo University.

After receiving the child in the OPD, the following procedures were carried out.

(1) Full history taking from the patients and/or parents.
(2) A questionnaire was given to the parents, which included questions regarding the full personal history of the child, the prenatal, perinatal, and postnatal history, past, and family history. Moreover, the patients were asked questions regarding the progressiveness of hearing loss to detect whether the hearing loss was rapidly (within 1 year after detecting hearing loss) or slowly progressive (over years after detecting hearing loss).
(3) Otoscopic examination.
(4) Basic audiological evaluation including:
  (a) Pure tone audiometry (PTA) for children older than 5 years, using an audiometer model Amplaid 455 with TDH 39 headphones, at frequencies 250–8000 Hz, for air conduction testing was performed. Bone conduction testing was carried out at frequencies 500–4000 Hz. The descending ascending method was used in these children.
  (b) Behavioral observational audiometry was used for children younger than 5 years to detect the degree of hearing thresholds in a sound-treated room using loudspeakers placed at an angle of $0°$ for binaural presentation, 1 m distant from the child. Warble tones were transmitted through the loudspeaker, at frequencies of 500, 1000, 2000, and 4000 Hz using the descending ascending method. The response noticed was mainly turning the head to the source of the sound.
  (c) Immittancemetry examination using an Amplaid 755, (Amplaid S.A.E., Italy) including tympanometry and acoustic reflex threshold (contralateral and ipsilateral), at frequencies of 500 Hz, 1, 2, and 4 KHz, was performed for each ear, in order to exclude middle ear diseases. Only patients with type A tympanogram were included in this study.
(5) Transient evoked otoacoustic emission (TEOAE) using an ILO 92 Otoacoustic emission otodynamic analyzer (Otodynamics, England) using a click stimulus (nonlinear) was performed in a sound-treated room; each ear was tested separately. The stimulus was elicited using a nonlinear click. The intensity was adjusted to ~ 80 dB sound pressure level.
Responses to 260 sets of stimuli were obtained within a time window of 20 ms starting 4 ms after stimulation. Responses to stimulus sets were averaged on each of two buffers (A and B). The average amplitude of these two wave forms represented the overall echo level in dB sound pressure level. In addition, by a simple cross correlation of the two wave forms, the whole reproducibility percent was computed. These two parameters were used in assessment of the TEOAE response. The results were considered to fulfill the criteria if the whole reproducibility percent was 50% or more and considered not to fulfill the criteria if the response was below this level [4].
(6) Auditory brainstem response (ABR) using an evoked response audiometer, Amplaid model 12 using a rarefaction click stimulus with a duration of 100 μs for threshold detection, at a repetition rate of 11 c/s, with a sweep number of 2000, was performed to assess the auditory pathway. Filter setting was 100–1500 Hz and the time window was 10 ms. The type of transducer used was a TDH 39 headphone and recording electrodes were silver silver-chloride cup-shaped electrodes. Wave V was elicited at 100 dB normalized hearing level (nHL), reduced in 10 dB steps till the threshold level was reached. If wave V was preserved down to 30 dB nHL, then the results were considered to be normal, whereas if wave V was found to be more than 30 dB nHL and corresponded to PTA thresholds at 2–4 kHz, response was considered to be present, and if wave V was not identified, then the response was considered to be absent.
(7) Computed tomography scan study to visualize the petrous bone in order to exclude congenital anomalies of the inner ear and internal auditory meatus was also performed.
(8) Genetic study and DNA screening in the genetic department was carried out to search for mutations involving *Cx26* using a PCR-based method and sequencing,

which was first carried out using both forward and reverse primers. Then, the PCR product was cleaned and followed again by repeating the PCR using both the forward and the reverse primers in separate tubes. Sequencing was then performed using an Applied Biosystems model 310 automated sequencer (Life Technologies Corporation, USA) to read the results, which were compared later with the published gene sequence.

As the child attended the genetic laboratory, a blood sample (5 ml) was drawn from him/her into an EDTA or a CBC tube and then genomic DNA was extracted from each sample using a 'Qiagen kit' (QIAGEN's Assay Technologies, Hamburg, Germany) according to the manufacturer's instructions. Allele-specific PCR amplification of genomic DNA was performed for the detection of the most common mutations of *Cx26* using mutation-specific PCR primers for rapid screening of cases and carriers. For detecting each mutation, three primers in two separate PCR reactions were used: one common reverse, one forward normal, and one forward mutant. Using 100 ng of DNA, 200 μmol/l dNTPs, 0.5 μ Taq polymerase, and 2.5 pmol of the common primers, samples were denatured at 95°C for 5 min, followed by 30–35 cycles at 95°C for 40 s, 60°C for 30 s, and 72°C for 30 s. PCR products were analyzed by electrophoresis in a 1.5% agarose gel containing ethidium bromide.

The following primers were used for 35delG:

(1) normal:
    TTGGGGCACGCTGCAGACGATCCTGGGGAG-3′
(2) mutant:
    TTGGGGCACGCTGCAGACGATCCTGGGGAT-3′
(3) common:
    GAAGTAGTGATCGTAGCACACGTTCTTGCA-3′

The following primers were used for the G2A bp71:

(1) normal:
    CAAACACTCCACCAGCATTGGAAAGATCGG-3′
(2) mutant:
    CAAACACTCCACCAGCATTGGAAAGATCGA-3′
(3) common:
    GAAGTAGTGATCGTAGCACACGTTCTTGCA-3′

Children who were found to be negative for the mutations were exposed to direct sequencing procedures. It is important to mention that the sequencing procedure is very expensive; therefore, it was more logical to start with the allele-specific PCR in order to reduce the number of patients undergoing direct sequencing.

PCR amplification of genomic DNA was performed with 40 ng of human DNA in each of four 8.4 μl PCR reactions, containing 1.25 μl PCR buffer (100 mmol/l Tris-HCL pH 8.8, 500 mmol/l KCL, 15 mmol/l MgCl, 0.01% w/v gelatin) and 200 mmol/l each of dATP, dCTP, dGTP, and dTTP.

The primers used in sequencing *Cx26* were:

CxG4352-μ/5′-TCG GCC CCA GTG GTA CAG-3′ (Mar.F)-forward;

Cx873-4 + /5′-CTG GGC AAT GCC TTA AAC TGG-3′ (Mar.R)-reverse;
Cx26A-μ (5′-TCT TTT CCA GAG CAA ACC GC-3′ (Mar. Int.F) internal primers-forward;
Cx637-μ (5′-CGA AGC CGC CTT CAT GTA CG3′ (Mar. Int.F) internal primer forward, and 0.25 U Taq polymerase.

Samples were denatured at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 30 s. PCR products from the four reactions were combined and cleaned using a QIA quick PCR purification kit (Qiagen, Chates-worth, California, USA), according to the manufacturer's instructions. PCR products were sequenced by dye primer sequencing using the forward and reverse primers and internal primers 5′.

Statistical analysis

Statistical analyses were carried out using Statistical Package for Social Sciences (SPSS) v.15.0 (IBM SPSS software for predictive analytics, Armonk, New York, USA). A P-value 0.05 or more was considered as insignificant, whereas a P-value less than 0.05 was considered as significant. Data were reported as numbers and percentages. The χ^2-test was used to assess the association between two qualitative variables. When the validity of the χ^2-test was violated due to small numbers, it was replaced by Fisher's exact test.

The data presented were of the whole study group (Tables and Figures), describing:

(1) Audiological findings (PTA, TEOAE, and ABR).
(2) Related factors: age groups, sex, familial (whether other members in the family are affected) versus sporadic (isolated case in the family); bilaterality (both ears affected) versus unilaterality (only one ear affected); rapidly progressive hearing loss (hearing loss was progressive within one year of detecting it) versus slowly progressive hearing loss (hearing loss was progressive within years after detecting it). Prelingual (hearing loss started before the age of language development) versus postlingual (hearing loss started after the age of language development).
(3) Different genetic results and the mutation detected.
(4) Relations between the findings of the genetic results with the audiological findings and the related mentioned factors are presented in tables and figures.

Results

A total of 100 children were chosen for the study from the Audiology clinic at Sheikh Salem Al-Ali center, age range 6 months to 18 years. For analysis, the study group was divided into three subdivisions: those younger than 6 years (41%), 6–10 years (36%), and older than 10 years (23%). 47% were men and 53% were women, with a men : women ratio of 1 : 1.13, which was statistically insignificant.

The results were analyzed separately in terms of ears. The degree of hearing loss among the study group is demonstrated in Table 1. The degree of hearing loss

Table 1 Distribution of hearing loss in the whole study group according to the degree of hearing loss

	N=100 (%)		
Degree of hearing loss (PTA)	Right ear	Left ear	Total
Mild to moderately severe	11 (11.0)	13 (13.0)	24 (12.0)
Mild to severe	6 (6.0)	5 (5.0)	11 (5.5)
Mild to profound	1 (1.0)	1 (1.0)	2 (1.0)
Moderately severe	25 (25.0)	21 (21.0)	46 (23.0)
Moderately severe to profound	5 (5.0)	7 (7.0)	12 (6.0)
Severe to profound	52 (52.0)	53 (53.0)	105 (52.5)

PTA, pure tone audiometry.

Table 2 Configuration of hearing loss in the study group

| Configuration	N (%)
Flat HL	148 (74.0)
High-frequency HL	15 (7.5)
Saucer-shaped HL	9 (4.5)
Steeply sloping HL	13 (6.5)
Left corner HL	13 (6.5)
Islands of HL	2 (1.0)

HL, hearing loss.

represents the grading of severity from the low frequencies (250 and 500 Hz) to the high frequencies (4 and 8 kHz). The moderately severe category represents the PTA average at 500 Hz, 1, and 2 kHz.

Different configurations of hearing loss were present in the study group. Flat SNHL was present in 148 ears out of 200 ears (74.0%). High-frequency SNHL was present in 15 ears (7.5%), steeply sloping SNHL was found in 13 ears (6.5%), and left corner SNHL was also found in 13 ears (6.5%). Saucer-shaped SNHL was present in nine ears (4.5%) and islands of HL were present in only two ears (1%) Table 2. TEOAEs were absent in all ears.

The percentages of different factors related to the hearing loss in the study group were evaluated. The familial factor showed a high percentage (57.0%), whereas the sporadic factor showed a percentage of 43.0%, which is also not low. Sixty two percent of children showed a history of rapidly progressive hearing loss, whereas 38% had a history of slowly progressive type of hearing loss. All children had bilateral hearing loss. Out of the 100 children, 70% were prelingual and 30% were postlingual (Fig. 1).

Regarding the genetic results, out of the 100 children, 15 showed positive results for *Cx26*; nine of these were heterozygous and six were homozygous. Eighty percent of the *Cx26* cases were due to the 35delG mutation. Out of the nine heterozygous children, six showed positive results for D1 (35delG) and three showed positive results for D2 (G2A at location base 71); all of the six homozygous children showed positive results for D1 (35delG) (Fig. 2).

Table 3 shows the relation between the positive and negative genetic results with the different degrees of hearing losses. As shown, for the mild to moderately severe degree of hearing loss, none of the ears showed

Figure 1
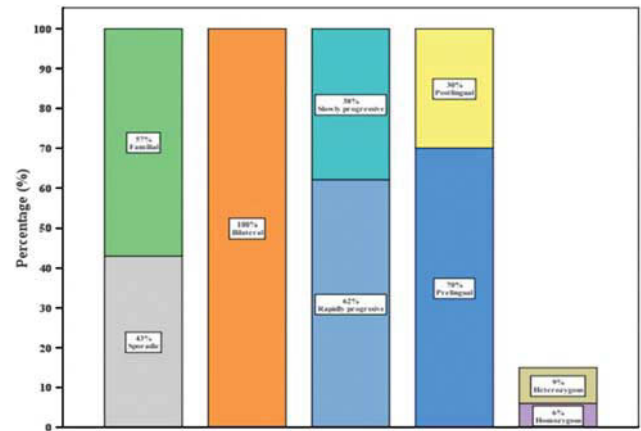
Factors related to the hearing loss in the study group.

Figure 2
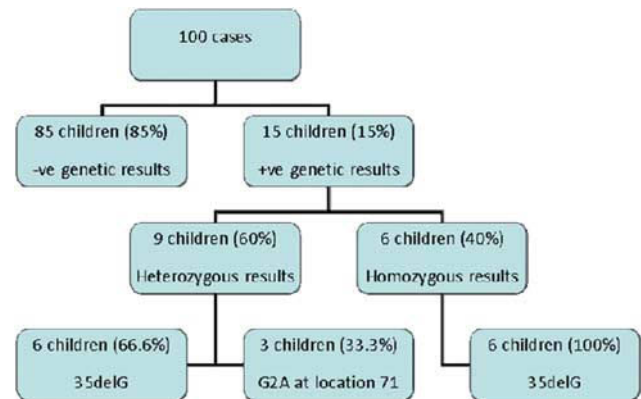
A schematic diagram showing a summary of the genetic results in the study group.

homozygosity. Similar results were obtained for the mild to severe degree of hearing loss. For the mild to profound results, none were homozygous or heterozygous. For the moderately severe degree, none were homozygous.

For the moderately severe to profound degree, none were homozygous or heterozygous.

For the severe to profound degree of hearing loss, 45 right ears (52.9%) showed negative genetic results, one ear (11.1%) showed positive findings for heterozygous, six (100%) for homozygous, whereas 45 left ears (52.9%) revealed negative genetic results, two left ears (22.2%) were positive for heterozygous, and six ears (100%) were homozygous.

The degree of hearing loss showed a significant difference ($P = 0.023$) according to the genetic results in the right ear. Among the right ears, all six homozygous ears had severe to profound hearing loss, whereas one (11.1%) heterozygous ear and 45 (52.9%) ears from the negative group had the same degree of hearing loss. Also, 22 (25.9%) from the negative group and three (33.3%)

Table 3 The relation of the degree of hearing loss to the genetic results in the study group

	Right ear N (%)			Left ear N (%)		
Degree	Negative	Hetero	Homo	Negative	Hetero	Homo
Mild to moderately severe	9 (10.6)	2 (22.2)	0 (0.0)	11 (12.9)	2 (22.2)	0 (0.0)
Mild to severe	3 (3.5)	3 (33.3)	0 (0.0)	4 (4.7)	1 (11.1)	0 (0.0)
Mild to profound	1 (1.2)	0 (0.0)	0 (0.0)	1 (1.2)	0 (0.0)	0 (0.0)
Moderately severe	22 (25.9)	3 (33.3)	0 (0.0)	17 (20.0)	4 (44.4)	0 (0.0)
Moderately severe to Profound	5 (5.9)	0 (0.0)	0 (0.0)	7 (8.2)	0 (0.0)	0 (0.0)
Severe to Profound	45 (52.9)	1 (11.1)	6 (100)	45 (52.9)	2 (22.2)	6 (100)
P-value		0.023			0.261	

P-values were generated using Fisher's exact test.

Table 4 The relation of the genetic results with the configuration of hearing loss in the study group

Configuration	+ ve GJB2 N=30	− ve GJB2 N=170	Total N=200
Flat HL	14	134	148
High-frequency HL	2	13	15
Saucer-shaped HL	4	5	9
Steeply sloping HL	4	9	13
Left corner HL	4	9	13
Islands of HL	2	–	2

HL, hearing loss; + ve, positive results; − ve, negative results.

Table 5 The relation of the auditory brainstem response findings to the genetic results in the study group

	Right ear N (%)			Left ear N (%)		
ABR response	Negative	Hetero	Homo	Negative	Hetero	Homo
Present	30 (35.3)	5 (55.6)	0 (0.0)	27 (31.8)	5 (55.6)	0 (0.0)
Absent	55 (64.7)	4 (44.4)	6 (100)	58 (68.2)	4 (44.4)	6 (100)
P-value		0.081			0.089	

P-values were generated using Fisher's exact test.
Present: ABR Wave V detected and corresponds to PTA thresholds at 2−4 KHz.
Absent: no response elicited.
ABR, auditory brainstem response; PTA, pure tone audiometry.

from the heterozygous group had a moderately severe degree of hearing loss. However, the degree of hearing loss was not statistically significant among the left ear according to the genetic results.

The *P*-value for the right ear was 0.023, which is statistically significant, especially for severe to profound SNHL, as all homozygous cases proved to have severe to profound SNHL and 52.9% of the negative genetic results had severe to profound SNHL; for the left ear, although the *P*-value was 0.261, which is insignificant statistically, all the homozygous (100%) and 52.9% of the negative genetic results had severe to profound SNHL, which is an important clinical finding.

The relation of the configuration of hearing loss with the positive and negative genetic results is demonstrated in Table 4. Among children who had positive GJB2 results, 14 ears had flat PTA, two ears had high-frequency SNHL, four ears had saucer-shaped PTA, four ears had steeply sloping PTA, four ears had left corner PTA, and two ears had islands of hearing loss.

The relation of the ABR results and the genetic findings in the study group is represented in Table 5. The *P*-values for the right ear and the left ear were 0.081 and 0.089, respectively. Correlation of the ABR results to the different genetic results was not statistically significant. It is important to mention that, had we only compared the homozygous and the heterozygous results without the negative genetic findings, the ABR results would have been significant, as the *P*-value would have been 0.044, which is statistically significant.

The correlation between age, sex, family history, onset, course of hearing loss and the genetic findings was

Table 6 The relation of the different factors to the genetic results in the study group

Factor	Negative N=85 (%)	Hetero N=9 (%)	Homo N=6 (%)	*P*-value
Sex				0.234
Male	41 (48.2)	2 (22.2)	4 (66.7)	
Female	44 (51.8)	7 (77.8)	2 (33.3)	
Age (years)				0.559
<6	36 (42.4)	4 (44.4)	1 (16.7)	
6–10	31 (36.5)	3 (33.3)	2 (33.3)	
>10	18 (21.1)	2 (22.2)	3 (50.0)	
Family history				0.023
Familial	44 (51.8)	7 (77.8)	6 (100)	
Sporadic	41 (48.2)	2 (22.2)	0 (0.0)	
Course of hearing loss				0.036
Rapidly progressive	53 (62.4)	3 (33.3)	6 (100)	
Slowly progressive	32 (37.6)	6 (66.6)	0 (0)	
Onset of hearing loss				0.017
Prelingual	61 (71.8)	3 (33.3)	6 (100)	
Postlingual	24 (28.2)	6 (66.7)	0 (0.0)	

P-values were generated using Fisher's exact test.

performed. The *P*-value comparing the sex factor to the different genetic results was 0.234, which was statistically not significant. Regarding age, the *P*-value was 0.559, which was not statistically significant. As regards the family history, the results were statistically significant (*P*-value was 0.023). The results regarding the course of hearing loss were found to be statistically significant, as the *P*-value was 0.036. As regards language development, all the homozygous cases were prelingual and the *P*-value was 0.017, which was statistically significant Table 6.

Discussion

Most of the studies carried out throughout the world on different ethnic groups have revealed that ARNSHL was the most common type of hearing loss and that the GJB2 mutation was the most common finding, mainly the 35delG [5]. Therefore, it was important to carry out this study on a Kuwaiti population to discover the common cause of hearing loss and the most common mutation as well.

Our study involved 100 children, their ages ranging from 6 months up to 18 years, presenting with ARNSNHL. The study showed no sex preference among the children.

The results demonstrated different degrees of hearing loss ranging from mild to profound SNHL among the study group. A large number had severe to profound SNHL. As regards the heterozygous group, the patients had a degree ranging from mild to moderate up to severe to profound SNHL, whereas the entire homozygous group had severe to profound SNHL. Cohn et al. [6] reported that the severity of deafness varied from mild–moderate to profound, even in patients homozygous for 35delG, and certain cases presented with progress in hearing loss.

The study group also demonstrated different configurations of hearing loss. Most of the children in the study group had the flat type of hearing loss, although a few had other configurations. Liu et al. [7] reported, from their study of the audiological features of GJB2 (Cx26) deafness, that the main audiogram shapes found were the residual/sloping (72.7%) and flat (23.4%). There was no difference in the severity and audiogram shapes of the hearing impairment between homozygous and compound heterozygous GJB2 deafness.

As regards the ABR results, the highest percentage of patients showed an absent response and the rest revealed a preserved response. All children in the study group demonstrated fail results on performing TEOAE. ABR and TEOAE results were consistent with the degree of hearing loss.

On comparing the familial factor with the sporadic one, the results demonstrated a higher percentage for the former; however, the latter was not low either. All of the children had bilateral SNHL. Most cases had rapidly progressive SNHL, whereas the rest had the slowly progressive type. The majority of cases were prelingual.

In this study, 35delG was the most common cause of ARNSNHL. Erbe et al. [8] provided the explanation that this mutation involves the deletion of a single guanine within a stretch of guanines at position 35, which results in a glycine-to-valine substitution at codon 12, followed by a premature stop at codon 13. It has been suggested that this common mutation may be due, in part, to mispairing during DNA synthesis. Our results suggest the 35delG/GJB2 mutation as the most common cause of ARNSNHL and this in agreement with other studies on different ethnic groups [5,9,10].

Pampanos et al. [11] demonstrated that out of 210 Greek patients, the 35delG mutation was responsible for 95.2% of the mutated alleles detected or 95% of deafness alleles in patients with biallelic GJB2 mutations. A total of 63 patients were homozygous for the 35delG mutation and 13 patients were heterozygous. Thirty five delG has accounted for the majority of the mutations detected in the GJB2 gene in white populations and is one of the most frequent disease mutations identified so far, with the highest carrier frequency of 3.5% in the Greek population [12].

A study carried out on 31 Egyptian families, with 83 participants ranging in age from 6 to 45 years, revealed that they all had prelingual severe to profound hearing loss. Only 14 (10%) of the tested participants were found to have a 35delG mutation; eight were homozygous and six were heterozygous. The latter demonstrated better hearing threshold levels by 5–10 dB than the former [13].

The analysis of the study carried out by Gazzaz et al. [14] on 116 Moroccan individuals showed that the 35delG mutation was the most common GJB2 allele variant, with a prevalence approaching 24%. Their data confirmed the importance of the GJB2 (essentially 35delG) mutation in hearing impairments. Another study on 25 nonrelated Moroccan children with nonsyndromic autosomal recessive hearing loss revealed that the 35delG mutation was responsible for almost half of the hearing loss among their patients (48%). There was no other GJB2 or GJB6 mutation among the rest of their patients [15]. Similar results on the prevalence of 35delG mutation were obtained by other investigators [16–18]. A study carried out by Walsh et al. [19] on 156 Palestinian families reported that hearing loss in 17 families (11%) was due to mutations in GJB2 (Cx26), a smaller fraction of GJB2-associated deafness than in other populations.

According to Ratbi et al., [15] mutations of the Cx26 gene are the most common cause of nonsyndromic autosomal recessive hearing loss. One of the GJB2 mutations, the 35delG, is recurrent in European and Mediterranean populations with an allelic frequency of at least 70% in patients with hearing loss caused by GJB2 impairment. Meguid et al. [20] reported that in their study, homozygosity was found in 100% of cases with the 35delG mutation.

Other researchers are in disagreement with our results. In an Asiatic population, the prevalence of this mutation was nil (0%), as in African Americans [21]. Wilcox et al. [22] reported that the prevalence was low in Australia (4%). Hamelmann et al. [23] reported that in patients from Ghana and West Africa, the prevalence was nil (0%). Gasmelseed et al. [24] stated that the prevalence in Sudan and Kenya was about 2.7%. These results could be explained by the fact that the chosen families may not have had the mutation in the entire coding region of the GJB2 gene. Also, they may have had mutations other than GJB2 that caused hearing impairments. One more possibility is that they may not have undergone the sequencing test after obtaining negative results by the PCR test, as it is an important test that has to be carried out if the results are found to be negative.

In terms of the factors related to genetic hearing loss in our study, the familial factor carried the highest percentage (57%), which is considered to be a significant factor in Kuwaiti populations. However, 43% sporadic cases were also found, which is also not low. Esmaeili *et al.* [25] reported in their study that consanguinity was present in 60% of the families and the frequency of 35delG was about 18.5% (49/266).

As regards the group of patients (15 children) with ARNSNHL, all six children with positive homozygous results who had the 35delG mutation showed a strong positive family history of hearing loss (100%), whereas out of the nine heterozygous children, seven (77.8%) showed a positive family history of hearing loss (six of them had the 35delG deletion, whereas one had the D2 deletion) and the remaining two (22.2%) children (who had D2) were found to be sporadic cases in the family.

Our study supported the findings of a previous study carried out in Kuwait, with the collaboration of Cairo University, which was carried on 500 elementary school children. The results showed that out of 500 school children, 3.3% had SNHL; the most common factor was the positive family history (50%), which in turn raises the high possibility of hereditary hearing loss [26].

All our 100 patients were found to have bilateral hearing loss (100%); 62% showed a rapidly progressive and 38% showed a slowly progressive pattern of hearing loss. All the homozygous patients had the rapidly progressive type of hearing loss, resulting in a severe to profound SNHL.

Out of the 100 children in our study, 70% were prelingual whereas 30% were postlingual. Considering the relation of the degree of hearing loss to the genetic results (homozygous vs. heterozygous), it was shown that children who were heterozygous showed different degrees of hearing loss ranging from mild to moderately severe SNHL to severe to profound SNHL. All six patients (100%) who were homozygous had severe to profound SNHL (all of them had rapidly progressive type); thus, they were all prelingual and did not develop speech. The statistical difference between the right and left ears as regards the relation of hearing loss to the genetic results may be attributed to the fact that not all patients had the same degree of hearing loss in both ears.

The major contribution of the autosomal recessive gene number 1 (DFNB1) to prelingual nonsyndromic recessive deafness suggests that many individuals in the general population are symptom-free carriers of the GJB2 mutation. Many researchers have determined the 35delG carrier, in which the highest carrier frequency has so far been found in Greeks and Italians, and this mutation seems to be particularly frequent in the Mediterranean population [27].

Denoyelle *et al.*, [9] analyzed over 100 patients and concluded that DFNB1 patients (considering DFNB1 patients as those who have two mutations in GJB2) are prelingually deaf, and present similar degrees of hearing loss for both ears, generating sloping or flat audiometric curves. The degree of deafness in their patients involved all ranges, but most patients had a severe to profound hearing impairment. Nevertheless, patients who were compound heterozygous for 35delG and another mutation were more commonly severely affected, while 35delG homozygous were predominant among profound or severely affected patients.

These results are in agreement with the majority of studies in which patients with mild and moderate hearing loss have rarely been reported. Previous reports have shown that deafness caused by the GJB2 mutation had a consistent pattern of hearing loss: prelingual, bilateral symmetrical, and usually severe to profound, with a wide variability in the extent of hearing loss [1,5,9–11,20,22, 28,29].

Gualandi *et al.* [30] attributed severe to profound hearing loss to a significant truncation of the protein after deletion. This observation may be explained by the complete absence of functional protein in recessive disorders, whereas in autosomal dominant disorders, dominant mutation may be consistent with initial function and subsequent hearing impairment due to a cumulative, degenerative process [20].

Engel-Yeger *et al.* [31] reported that GJB2 mutations cause variable degrees of audiometric hearing loss, ranging from moderate to profound, among mutations of 35delG. Morton [32] reported that generally, patients with an autosomal recessive hearing impairment have prelingual and congenital deafness.

Arjan *et al.* [33] reported that the incidence of prelingual hearing loss is about one in 1000 newborns in Western Europe. The nonsyndromic forms of deafness account for 70% of these cases, of which 85% are autosomal recessively inherited. Iliades *et al.* [12] concluded that the 35delG GJB2 mutation is responsible for one-third of prelingual SNHL in deaf Greek populations, which is higher than that usually reported for the White population (20%).

Mustapha [13] reported in his study on an Egyptian population that the prevalence of inherited prelingual hearing loss is among the highest in the world. This could be attributed to the extended consanguineous marriages in the Egyptian community. The results of our study agreed with the study of Kolkaila *et al.* [34], who examined 27 SNHL patients with a family history of hearing loss for *Cx26* gene mutations. The percentage of positive mutations in those patients was 70.4%. Four types of *Cx26* gene mutations were found in these patients: 35delG, R143W, V271 mutation, and 235delG. The 35delG mutation was the most frequent type as it was detected in 78.9% of these patients. Moreover, 70% of the cases with this mutation were homozygous.

Mahdieh *et al.* [5] reported that the study carried out on the Kurdish population of Iran, on 86 families with ARNSNHL, revealed that all deaf persons in these families had PTA consistent with severe to profound deafness.

Although 30 genes responsible for nonsyndromic hearing loss (NSHL) have been discovered, the *Cx26* gene (GJB2) is commonly found in unknown origins of

prelingual deafness and accounts for 20–50% of prelingual deafness in various ethnic groups [35].

Liu *et al.* [7] carried out a study on 399 individuals with nonsyndromic deafness for mutations in the *Cx26* gene (GJB2) by sequence analysis to characterize audiological profiles. A total of 77 (19%) of the deaf individuals had biallelic GJB2 mutations (either homozygous or compound heterozygous mutations; GJB2 deafness). All categories of hearing loss severity were found. Eighty-one percent of patients with GJB2 mutations had severe to profound hearing loss; 18.4% had mild to moderate hearing loss.

Marlin *et al.* [16] reported that hearing impairment was frequently less severe in compound heterozygotes than in 35delG homozygotes. Moderate or mild hearing impairment was more frequent in patients with one or two noninactivating mutations than in patients with two inactivating mutations. Of 93 patients, hearing loss was stable in 73, progressive in 21, and fluctuant in two. Progressive hearing loss was more frequent in patients with one or two noninactivating mutations than in those with two inactivating mutations. In 49 families, hearing loss was compared between siblings with similar genotypes and variability in terms of severity was found in 18 families (37%). They concluded that the genotype may affect deafness severity, but environmental and other genetic factors may also modulate the severity and evolution of GJB2–GJB6 deafness.

Iliades *et al.* [12] performed PTA in all Austrian individuals in their study and reported severe to profound SNHL. A clear cause of hearing loss was the heterozygous alterations 35delG/del311-324 and 35delG/L90P. In three hearing loss cases that were not identified during initial OAE testing, homozygous 35delG and 35delG/R184P defined the genetic basis for hearing loss in two cases, whereas one case had wild-type GJB2. They concluded that the high mutation rate in the Austrian population, especially in neonates identified during the newborn screening program, confirms the importance of screening for mutations in GJB2.

Ramserbner *et al.* [36] carried out a study on 21 neonates with NSHL. They were identified by postpartum OAE and ABR. GJB2 testing was performed by direct sequencing. Mutations in GJB2 were found in 15 of 21 children (71.4%) identified by neonatal audiological screening. The 35delG mutation in GJB2 was found to be homozygous in 10 cases (47.6%).

Orzan *et al.* [10] reported a high prevalence of the 35delG mutation and a large proportion of prelingual hearing impairments and this argued for modification of the protocols used to investigate the etiology of childhood hearing impairment. Early screening of the *Cx26* mutation in all patients with nonsyndromic familial and sporadic permanent childhood hearing impairment seems to be justified.

Kudo *et al.* [35] reported that early diagnosis of deafness in newborn babies and prompt training for language development is mandatory and must depend on DNA extracted from IsoCode and real-time quantitative PCR within 45 min, which is sufficient to detect the full sequence of GJB2, where the four types of common GJB2 mutations are reliably detected within 2.5 h.

We concluded from our study that the prevalence of hereditary hearing loss is high due to frequent positive consanguinity among Kuwaiti children. The *Cx26* mutation percentage was 15%. The most common mutation was the 35delG (80%), which is considered to be high and agrees with the results of most studies carried out on other ethnic groups. The homozygous group demonstrated a more severe clinical picture than the heterozygous group, which presented with a wide variation in the degree of hearing loss. All homozygous children were found to have a prelingual severe to profound SNHL. There was no sex preference in the population of children under study. Development of a neonatal hearing screening program, in addition to screening and follow-up of children at risk of developing hearing loss and children at school entry (with repeated annual rescreening), is recommended. This would ensure timely management and hence increase the child's chance of having a healthy and productive life.

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