Non-Syndromic Autosomal Recessive Deafness in Gaza Strip: A study on Selected Connexin 26 Gene Mutations

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Non-Syndromic Autosomal Recessive Deafness in Gaza Strip: A study on Selected *Connexin 26* Gene Mutations

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نتيجة الحكم على أطروحة ماجستير

بناءً على موافقة شئون البحث العلمي والدراسات العليا بالجامعة الإسلامية بغزة على تشيك لجنة الحكم على أطروحة الباحثة/ بديرة فؤاد موسى السماك لنيل درجة الماجستير في كلية العلوم قسم العلوم الحياتية - تحاليل طبية وموضوعها:

دراسة الطفرات المتعلقة في جين الكونكسين 26 المسؤول عن الصمم الوراثي المنتحي الغير المرتبط بمتلازمة في قطاع غزة

Non-Syndromic Autosomal Recessive Deafness in Gaza Strip: A study on Selected Connexin 26 Gene Mutations

وبعد المناقشة التي تمت اليوم السبت 02 شعبان 1435 هـ الموافق 31/05/2014م الساعات الحادية عشرة صبحاً، اجتمعت لجنة الحكم على الأطروحة والمكونة من:

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وقد اشتملتيجيتي على الملاحظات المأتية]

واللجنة إذ تمنحها هذه الدولة فإنها توصيني بتقوى الله وزوج طاعته وأن تسخر علمها في خدمة بينها وبيئها.

والله والتحية

مساعد نائب الرئيس للبحث العلمي و للدراسات العليا

أ.د. فؤاد علي العمري
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Badria F. Essammak

May, 2014

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Abstract

Hearing loss (HL) is a common and highly heterogeneous sensory disorder which affects approximately 1 in 1000 infants. Genetic causes are thought to be responsible for more than 60% of the cases with the majority of non-syndromic HL being inherited in an autosomal recessive pattern. The gene first identified in 1997 to be associated with non syndromic autosomal recessive deafness (NSARD) was Gap junction beta 2 (GJB2) which codes for Connexin 26 (Cx26). Gap junction protein belongs to a family of transmembrane proteins with about 20 members in humans. Cx26, has been shown to play a key role in potassium homeostasis, which is essential for sound transduction.

The aim of this study was to study the mutations in Cx26 gene in families with NSARD in Gaza strip.

Descriptive analytical design was used to conduct this study and samples selected from three HL institutions in Gaza strip.

The patients were screened for five GJB2 gene mutations namely, 35delG, 167delT, IVS1+1G>A, W77R and 235delC. First, the 35delG and 167delT mutations were screened using restriction enzyme analysis of the corresponding PCR products. Second, when no 35delG or 167delT mutation was detected, screening was continued for the IVS1+1G>A, 235delC and W77R mutations.

Our results revealed that GJB2 mutations contributed to 44.3% of the NSARD with allele frequency of 0.4%. The mutations were identified in either heterozygous or homozygous form. The most frequently encountered mutation was 35delG which accounted for 35.7% of the NSARD in either heterozygous (1.4%) or homozygous (34.3%) and represented about 80.5% of all mutations detected in this study. The second detected mutation was 235delC which accounted for 8.6% of NSARD and was recorded in only heterozygous form. The third mutation was IVS1+1G>A which was identified in only one proband (1.4%) in a compound heterozygous form along with 35delG, Whereas, 167delT and W77R were not observed in our samples.

We concluded that there is a significant contribution of GJB2 mutations to congenital NSARD in the Palestinian population of Gaza strip. Screening for GJB2
mutations particularly, 35delG, 235delC and IVS1+1G>A should be offered to NSARD patients to confirm diagnosis of their congenital deafness, to deliver proper genetic counseling for the affected individuals and their families and to provide the patients the optimal management.

**Keywords:** NSARD, Cx26, GJB2, Gaza strip.
دراسة الطفرات المتعلقة في جين الكونكسين 26 المسؤول عن الصمم الوراثي المتنحي الغير مرتبط بمتلازمة في قطاع غزة

المتخصّص

فقدان السمع هو خلل حسي شائع متعدد الأسباب حيث يصيب حوالي 1 لكل 1000 من المواليد سنوياً، ولكن 60% من الحالات تعزى لأسباب وراثية وخاصة فقدان السمع الغير مرتبط بمتلازمة والذي يورث بصورة متجهية.

في سنة 1997 تم تحديد أول جين له علاقة قوية بفقدان السمع المتنحي الغير مرتبط بمتلازمة وهو جين التدخلات الفجوة بيتا 2 (GJB2) والذي يشفر البروتين 26. هذا البروتين يُعتبر عن ممرات اتصال ينتمي لعائلة كبيرة من البروتينات عبر الشبكة والتي تتكون من 20 بروتين. البروتين 26 يلعب دور كبير في المحافظة على مستوى ملح اليودوسوم في الفص العصبي والذي يلعب دوراً مهماً في توصيل النواحي الصوتية وتحويلها إلى نبضات كهربائية ترسل عبر العصب السمعي إلى مركز السمع بالدم.

تهدف هذه الدراسة إلى دراسة الطفرات المؤثرة في جين الكونكسين 26 في العائلات المصابة بالصمم الوراثي المتنحي في قطاع غزة.

وهذه الدراسة من النوع الوصفي التعميمي، وتم جمع العينات فيها من ثلاثة مؤسسات لمصم في قطاع غزة.

تم فحص عظام المرضى لدراسة خمسة أنواع من الطفرات وائب 35delG و167delT و235delC وIVS1+1G>A وW77R. حيث أن في البداية تم البحث عن الطفرتين 35delG و167delT باستخدام تقنية التقسيط الانزيمي لنتائج الحمض النووي المتضخمة (PCR). ثانياً في حالة عدم وجود الطفرتين استمر البحث عن الطفرات الثلاثة الأخرى.

أظهرت نتائج هذه الدراسة بأن الطفرات المؤثرة في جين GJB2 ساهمت بنسبة 44.3% ونسبة وجود الأليل الخاص بهذه الجين كانت 0.4% وهذه الطفرات موجودة إما على هيئة تماثل النواتج (homozygous) أو تمثل نسبة محلية. واثبت هذه الطفرات شبيهة في قطاع غزة هي 35delG حيث وجدت على هيئة تماثل النواتج بنسبة 35.7% ونسبة وجود الأليل الخاص بنسبة 0.4%. واثبت هذه الطفرات شبيهة في قطاع غزة هي 235delC حيث وجدت على هيئة تماثل النواتج بنسبة 8.6% بينما ثالث طفرة شبيهة في قطاع غزة كانت IVS1+1G>A حيث وجدت في مريض واحد فقط بنسبة 1.4% على هيئة IVS1+1G>A و167delT وW77R.

في النهاية، لقد استنتجنا وجود مساهمة كبيرة لطفرات جين GJB2 في الصمم الوراثي المتنحي الغير مرتبط بمتلازمة في قطاع غزة. لذلك، فصح هذه الطفرات خاصة في حالات إجراء على الأطفال الذين يعانون من هذا المرض مما يساعد في الكشف المبكر عن هذا المرض وتقدم الاستشارة الوراثية المناسبة للأطفال الذين يعانون من هذا المرض وتقدم الاستشارة الوراثية المناسبة للأطفال الذين يعانون من هذا المرض.

الكلمات المفتاحية: فقدان السمع المتنحي الغير مرتبط بمتلازمة، كونكسين 26، جين التدخلات الفجوة بيتا 2، قطاع غزة.
Dedication

To my beloved parents

My brother and sisters

My wonderful husband, daughter

My teachers and friends

To Islamic University in Gaza

To all deaf people in the world
I have many people to thank for their help to complete this thesis:

First of all, I deeply thank my supervisor, Prof. Fadel A. Sharif for the time, support and interest that have made the writing of this thesis possible and, whose support was vital for me to overcome all the difficulties I faced.

My special thanks go to my colleagues at the Genetic Diagnosis lab of the Islamic University of Gaza, especially Mr. Mohammmed Ashour, who worked hard to complete the practical part of this thesis.

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I would like to express my sincere thanks to Gaza Strip hearing loss institutions and centers, especially Mustafa Sadiq Rafii Secondary School for the Deaf, Atfaluna Society for the Deaf Children, and Basma Center for Audiology & Speech Therapy.

Special gratitude is offered to all the deaf participants and their families, for their cooperation in my study.

My family deserves a special mention, who encouraged me all along the way to complete this work, especially my father Mr. Fouad Essammak, Who helped me during blood collection from patients. Also thanks to my mother, brother, and sisters.

Finally, my sincere love and respect to my husband for his support and encouragement, Also, my daughter, thank you for waiting.
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<tr>
<th>Abbreviation</th>
<th>Term description</th>
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<tbody>
<tr>
<td>167delT</td>
<td>Deletion of a T at position 167 of <em>GJB2</em></td>
</tr>
<tr>
<td>235delC</td>
<td>Deletion of a C at position 235 of <em>GJB2</em></td>
</tr>
<tr>
<td>35delG</td>
<td>Deletion of a G at position 35 of the <em>GJB2</em></td>
</tr>
<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>ApaI</td>
<td>Restriction enzyme from <em>Acetobacter pasteurianus</em></td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BstI</td>
<td>Restriction enzyme from <em>Bacillus stearothermophilus</em></td>
</tr>
<tr>
<td>C</td>
<td>Cytosine</td>
</tr>
<tr>
<td>CHL</td>
<td>Conductive hearing loss</td>
</tr>
<tr>
<td>cM</td>
<td>Centimeter</td>
</tr>
<tr>
<td>Cx26</td>
<td>Connexin 26</td>
</tr>
<tr>
<td>Cx30</td>
<td>Connexin 30</td>
</tr>
<tr>
<td>dB</td>
<td>Decibels</td>
</tr>
<tr>
<td>del</td>
<td>Deletion</td>
</tr>
<tr>
<td>DFNA</td>
<td>Deafness, autosomal dominant</td>
</tr>
<tr>
<td>DFNB</td>
<td>Deafness, autosomal recessive</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine Tetraacetic Acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>F</td>
<td>Forward</td>
</tr>
<tr>
<td>G</td>
<td>Guanine</td>
</tr>
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<td>GJB2</td>
<td>Gap junction beta 2</td>
</tr>
<tr>
<td>GJB6</td>
<td>Gap junction beta 6</td>
</tr>
<tr>
<td>GJIC</td>
<td>Gap junction mediated intercellular communication</td>
</tr>
<tr>
<td>GJs</td>
<td>Gap junctions</td>
</tr>
<tr>
<td>hCx26</td>
<td>Human connexin 26</td>
</tr>
<tr>
<td>HID</td>
<td>Hystrix-like ichthyosis with deafness</td>
</tr>
<tr>
<td>HL</td>
<td>Hearing loss</td>
</tr>
<tr>
<td>HphI</td>
<td>Restriction enzyme from <em>Haemophilus parahaemolyticus</em></td>
</tr>
<tr>
<td>IHCs</td>
<td>Inner hair cells</td>
</tr>
<tr>
<td>ISO</td>
<td>International Organization for Standardization</td>
</tr>
<tr>
<td>IVS</td>
<td>Intervening Sequence</td>
</tr>
<tr>
<td>IVS1+1G&gt;A</td>
<td>G changed to A one nucleotide from the beginning of exon 1 or G changed to A at −3172</td>
</tr>
<tr>
<td>K+</td>
<td>Potassium ion</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>KDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>KHz</td>
<td>Kilohertz</td>
</tr>
<tr>
<td>KID</td>
<td>Keratitis-ichthyosis-deafness</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Term description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------</td>
</tr>
<tr>
<td>MHL</td>
<td>Mixed hearing loss</td>
</tr>
<tr>
<td>MspI</td>
<td>Restriction enzyme from <em>Moraxella</em> species</td>
</tr>
<tr>
<td>MwoI</td>
<td>Restriction enzyme from <em>Methanobacterium wolfeii</em></td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>NSADD</td>
<td>Non syndromic autosomal dominant deafness</td>
</tr>
<tr>
<td>NSARD</td>
<td>Non-syndromic autosomal recessive deafness</td>
</tr>
<tr>
<td>NSHL</td>
<td>Non syndromic hearing loss</td>
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<tr>
<td>OHCs</td>
<td>Outer hair cells</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PCR-RFLP</td>
<td>Polymerase chain reaction-Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>PGD</td>
<td>Preimplantation genetic diagnosis</td>
</tr>
<tr>
<td>PPK</td>
<td>Palmoplantar keratoderma</td>
</tr>
<tr>
<td>PSDM</td>
<td>PCR-mediated site directed mutagenesis</td>
</tr>
<tr>
<td>q</td>
<td>Chromosome’s long arm</td>
</tr>
<tr>
<td>R</td>
<td>Arginine amino acid</td>
</tr>
<tr>
<td>R</td>
<td>Reverse</td>
</tr>
<tr>
<td>SNHL</td>
<td>Sensorineural hearing loss</td>
</tr>
<tr>
<td>T</td>
<td>Thymine</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>W</td>
<td>Tryptophan amino acid</td>
</tr>
<tr>
<td>W77R</td>
<td>Change of tryptophan at position 77 into arginine</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>wt</td>
<td>Wildtype</td>
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<td>α</td>
<td>Alpha</td>
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<td>Beta</td>
</tr>
<tr>
<td>γ</td>
<td>Gamma</td>
</tr>
<tr>
<td>μL</td>
<td>Microliter</td>
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Chapter 1
Introduction

1.1 Overview

Hearing loss (HL) is the most prevalent form of sensory impairment in humans, with approximately 1 in 1000 infants being born with a serious hearing deficit (Shahin et al., 2002). Hereditary HL (HHL) can be subdivided into two types: syndromic or nonsyndromic. The syndromic type is associated with other distinctive clinical features beyond deafness and accounts for 30% of HHL. The nonsyndromic type, in which HL is the only clinical manifestation, represents the other 70% (Matsunaga, 2009). Regarding nonsyndromic HL, autosomal recessive is the most frequent inheritance pattern, accounting for 75-85% of the cases (Snoeckx et al., 2005; Ibrahim et al., 2011). It is followed by dominant pattern (12-13%) and X-linked or mitochondrial inheritance that accounts for 2-3% of the cases (Ibrahim et al., 2011). Previous studies defined at least 23 human nonsyndromic autosomal recessive deafness (NSARD) loci (Petersen and Willems, 2006).

The different loci for the numerous forms of deafness have been called DFN (for deafness) and are numbered in chronological order of discovery. The first NSARD locus, DFN1 was identified by Guilford et al in 1994 which accounts for at least 50% of this type of HL (Mukherjee et al., 2003). At chromosome 13q11-q12, DFN1 locus comprises Gap Junction β-2 (GJB2) and Gap Junction β-6 (GJB6) genes that code for connexin 26 (Cx26) and connexin 30 (Cx30), respectively (Rodríguez-Paris et al., 2011).

Connexin 26 (Cx26) is one member of a family of related gap-junction channel forming proteins (Mukherjee et al., 2003). These gap junction proteins oligomerize as hexamers to form transmembrane channels called connexons. Connexons from the cell membranes interdigitate to form direct intercellular communications pathways, the gap junction channels (RamShankar et al., 2003). Some forms of connexin are expressed in a wide variety of tissue, whereas others are restricted to specific cell types. Turnover of connexins appears to be rapid, suggesting that the levels of most forms are dependent on transcription or mRNA turnover (Kelley et al., 1998).
Human connexins are classified either by molecular mass (26-59 kDa) or by sequence similarities into 3 groups: gap junction α (GJA), gap junction β (GJB) or gap junction γ (GJC). NSARD is caused by mutations in the gap junction genes encoding the β connexins (Mukherjee et al., 2003).

*GJB2*, the gene encoding human Cx26 (hCx26), was the first gene to be linked to an autosomal recessive form of deafness, *DFNB1* (Zonta et al., 2012). *GJB2* gene has 5500 bp and a single coding exon, in a total of two exons. More than 200 different pathogenic mutations were identified in this gene that account for 10-40% of congenital HL (Falah et al., 2011; Tang et al., 2006). *GJB2* mutation spectrum differs among various populations or even within a particular country. Therefore, it is necessary to establish the frequency of *GJB2* mutations in any population (Bonyadi et al., 2011).

Mutations at the *DFNB1* locus can be classified in two groups: (i) those that affect the coding sequence of *GJB2*, and (ii) those that lie outside the coding sequence of *GJB2* and affect the expression and/or regulation of this gene (Gandía et al., 2013).

1.2 Objectives

1.2.1 General Objective

To study the mutations in Cx26 gene in families with NSARD in Gaza strip.

1.2.2 Specific Objectives

1. To perform screening for 5 mutations in Cx26 in two steps. First, the 35delG and 167delT mutations are screened in the deaf patients. Second, when no 35delG or 167delT mutation is detected, screening is continued for the 235delC, IVS1+1G>A and W77R mutations.

2. To identify the prevalent *GJB2* mutations contributing to NSARD in Gaza strip for future development of diagnostic tests.

3. To compare the prevalence of detected mutations in Gaza strip with those recorded in neighboring countries.
1.3 Significance

Due to the high rate of consanguineous and assortative marriages, NSARD is a prevalent genetic disorder in Gaza strip. Hitherto, no previous studies investigated the gene mutations linked to NSARD in Gaza strip. Screening for mutations in Cx26 gene will be useful to identify the cause of deafness, develop suitable diagnosis and deliver appropriate genetic counseling to NSARD families. Moreover, through seeking preimplantation genetic diagnosis (PGD), families with known mutations may have the possibility of having children free from those mutations.
Chapter 2

Literature Review

2.1 Deafness

Deafness is a sensory impairment which results in a partial or total loss in reception of sound. The intensity of sound can be measured in decibels (dB). It is usual to assess hearing thresholds at frequencies of 0.25, 0.5, 1, 2, 4 and 8 KHz. Sounds of each frequency are presented at different intensities to a subject and the response is recorded graphically as an audiogram. A loss in hearing is indicated if the threshold for perception of sound for any frequency is elevated by 10 dB or greater as compared to the defined standard value for each frequency (Naz, 2012).

2.2 Anatomy and physiology of the inner ear

The ear is located within the temporal bone and consists of three main structural components: the outer ear, the middle ear, and the inner ear. The ear’s main functions are amplification, transduction and encoding of the external mechanical input (sound) to an electrochemical output traveling to the brainstem nuclei and cortex. The outer and middle ears comprise the sound conductive system and are both gas filled compartments (Schuknecht, 1993).

The inner ear, which resides in a bony cavity, is a fluid-filled organ composed of two major regions, the cochlea, which processes the auditory signal and the vestibular apparatus (utricle, saccule, and ampullae of the semicircular canals) which helps maintain balance by responding to gravity and acceleration (Figure 2.1) (Davis, 2003).

The processing of auditory signals takes place in the cochlea, which is a bony canal that coils around it's central core called the modiolus, having 2.75 turns in humans.

The cochlea subdivided into three large cavities, the scala vestibule and the scala tympani. Both of these cavities contain a liquid called perilymph that is similar in composition to cerebrospinal fluid. A third cavity, the scala media (cochlear duct), lies between the scala vestibuli and the scala tympani. The scala media, is a triangular membranous canal that is limited by the Reissner's membrane (top), the basilar membrane (bottom) and stria vascularis (strial abneural side), and contains the endolymph fluid (Davis, 2003).
The molecular as well as physical and biological properties of the cochlear duct vary throughout its length and are reflected in differences and gradients of gene expression (Davis, 2003).

The stria vascularis is an ion transporting epithelium composed of three cell types: marginal, intermediate, and basal cells. Together, they generate the high endolymphatic potassium ion concentration and positive endocochlear potential. The organ of Corti, which is the sensory transduction organ of the cochlea, rests just on top of the basilar membrane throughout the cochlear duct. The organ of Corti is comprised of hair cells, supporting cells, neurons, blood vessels and the tectorial membrane (Kessel and Kardon, 1979; Schuknecht, 1993).

The supporting cells include several different types of cells, most of which have a poorly understood function. It has been shown that the supporting cells function to recycle the potassium ions, form a sealed barrier between the endolymph and perilymph filled spaces, maintain the hair cell function, and structurally support the organ of Corti, specifically as the hair cells are in contact with supporting cells. The supporting cells are
coupled to each other by gap junctions (comprised of connexins 26, 30, 31 and 43, in the cochlea, and 26, 30 and 43, in the vestibular system), allowing them to act as a 'functional unit' (Forge et al., 2003).

The hair cells of the cochlea are the sensory cells. At their apical surface lie bundles of stiff-actin filled microvilli, named stereocillia (Pickles et al., 1984; Furness and Hackney, 1985).

The hair cells of the cochlea are divided into inner and outer hair cells (IHCs and OHCs) that differ in their structure, pattern of innervation and function (Snoeckx et al., 2005).

The OHCs translate the changes in membrane potential into macroscopic changes in the length of their cell bodies, which is mediated by Prestin, a transmembrane protein located in the cell membrane of the outer hair cells (Zheng et al., 2000). This generates the mechanical energy that is required for amplifying the sound induced vibrations in the cochlea, which are largely responsible for hearing sensitivity and frequency resolving capability of the ear (Dallos and Fakler, 2002).

By contrast, IHCs function as the sensory receptors of the hearing organ and convey essentially all auditory information to the brain (Ottersen et al., 1998).

2.3 Grades of HL

HL may present itself with variable grades which are adapted from World Health Organization (WHO) in 2014 as described in (Table 2.1). Grades 2, 3 and 4 are classified as disabling hearing impairment. The audiometric International Organization for Standardization (ISO) values are averages of values at 500, 1000, 2000, 4000 Hz (WHO, 2014).
Table 2.1 Grades of HL (WHO, 2014).

<table>
<thead>
<tr>
<th>Grade of impairment</th>
<th>Corresponding audiometric ISO value</th>
<th>Performance</th>
<th>Recommendation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0- Non</td>
<td>25 dB or better (better ear)</td>
<td>No or very slight hearing problems. Able to hear whispers.</td>
<td>-</td>
</tr>
<tr>
<td>1- Slight (Mild)</td>
<td>26-40 dB (better ear)</td>
<td>Able to hear and repeat words spoken in normal voice at 1 meter.</td>
<td>Counseling. Hearing aids may be needed.</td>
</tr>
<tr>
<td>2- Moderate</td>
<td>41-60 dB (better ear)</td>
<td>Able to hear and repeat words spoken in raised voice at 1 meter.</td>
<td>Hearing aids usually recommended.</td>
</tr>
<tr>
<td>3- Severe</td>
<td>61-80 dB (better ear)</td>
<td>Able to hear some words when shouted into better ear.</td>
<td>Hearing aids needed. If no hearing aids available, lip-reading and signing should be taught.</td>
</tr>
<tr>
<td>4- Profound including deafness</td>
<td>81 dB or greater (better ear)</td>
<td>Unable to hear and understand even a shouted voice.</td>
<td>Hearing aids may help understanding words. Additional rehabilitation needed. Lip-reading and sometimes signing essential.</td>
</tr>
</tbody>
</table>

2.4 Classification of HL

HL can be classified as conductive, sensorineural, or mixed HL, based on the anatomic location of the problem (site of lesion, i.e., middle or inner ear). HL may also be labeled as unilateral or bilateral, depending on whether the loss is in one (unilateral) or both (bilateral) ears. The degree of loss might be the same in both ears (symmetrical HL) or it could be different for each ear (asymmetrical HL) (Shemesh, 2010).

Conductive HL (CHL) is characterized by an obstruction to air conduction that prevents the proper transmission of sound waves through the external auditory canal and/or the middle ear. It is marked by an almost equal loss of all frequencies. CHL may be congenital or caused by trauma, severe otitis media, otosclerosis, neoplasms, or atresia of the ear canal. Some CHL can be treated surgically with tympanoplasty or stapedectomy, and the use of hearing aids and assistive listening devices may also be beneficial (Shemesh, 2010).
Sensorineural HL (SNHL) occurs when the sensory receptors of the inner ear are dysfunctional. SNHL is a lack of sound perception caused by a defect in the cochlea and/or the auditory division of the vestibulocochlear nerve. This type of HL is more common than CHL and is typically irreversible. It tends to be unevenly distributed, with greater loss at higher frequencies. SNHL may result from congenital malformation of the inner ear, intense noise, trauma, viral infections, ototoxic drugs (e.g., cisplatin, salicylates, loop diuretics), fractures of the temporal bone, meningitis, cochlear otosclerosis, aging (i.e., presbycusis), or genetic predisposition, either alone or in combination with environmental factors. Many patients with SNHL can be habilitated or rehabilitated with the use of hearing aids. Patients with profound bilateral SNHL (e.g., at least 90 dB) who derive no benefit from conventional hearing aids may be appropriate candidates for the cochlear implant device, which bypasses the damaged structures of the cochlea and stimulates the function of the auditory nerve. Auditory brainstem implants, which are similar to multichannel cochlear implants, are used in patients with neurofibromatosis type 2 following vestibular schwannoma removal, especially those individuals who have lost integrity of the auditory nerves (Shemesh, 2010).

Individuals with mixed HL (MHL) have both conductive and sensory dysfunction. MHL is due to disorders that can affect the middle and inner ear simultaneously, such as otosclerosis involving the ossicles and the cochlea, head trauma, middle ear tumors, and some inner ear malformations. Trauma resulting in temporal bone fractures may be associated with CHL, SNHL and MHL (Shemesh, 2010).

### 2.5 Mode of Inheritance

The pattern of inheritance of monogenic prelingual non-syndromic deafness may be an autosomal dominant, autosomal recessive, X-chromosomal recessive or maternal trait (Smith and Hone, 2003). The percentage of prelingual HHL transmitted through an autosomal recessive trait is 75-80%, an autosomal dominant trait is 10-20%, X-linked is 1-5%, or maternally inherited is 0-20% depending upon the investigated population (Petersen, 2002; Smith and Hone, 2003).

NSARD is usually prelingual and stable and it is very rare to be post-lingual deafness. The autosomal recessive forms are usually more severe than the other forms and are
entirely due to cochlear defects. While non syndromic autosomal dominant deafness (NSADD) is commonly post-lingual and progressive (Schrijver, 2004).

In a recessive inheritance, both inherited alleles have to be mutated to cause HL. The person is referred to as "Homozygous" or "heterozygous" for the mutation. The person may be "compound heterozygous" for the mutation. Carriers do not have HL (Bayazit and Yilmaz, 2006).

The genotype-to-phenotype relation in HHL is important. In terms of HL, phenotype is the type of HL that can be figured out by means of an audiogram. Genotype means the mutation in the gene resulting in HL. The responsible genotype alteration or mutation can be better understood by looking at the phenotype or the type of HL (Bitner-Glindzicz, 2002).

Prefix DFN is derived from the term "deafness" and it is used to describe the locus responsible for genetic non-syndromic HL (NSHL). The abbreviation such as DFNA is used to present the autosomal dominant form, DFNB to the autosomal recessive form of HL, and DFN also represents X-linked NSHL. Numbers added after these abbreviations, like DFNB1 or DFNB30, represent the sequential order in which the loci are found (Bayazit and Yilmaz, 2006).

2.6 Gap junction protein (connexin protein) structure and assembly

Gap junctions (GJs) or connexins are highly specialized membrane structures that contain clusters of channels. This organization requires the membranes of two neighboring cells to come close to each other leaving a 2-4 nm gap (Bruzzone et al., 1996).

Connexins have a highly conserved form of transmembrane domains separating two extracellular loops from a middle cytoplasmic loop and the N- and C-terminal cytoplasmic ends (RamShankar et al., 2003). Connexins oligomerize with five other connexin molecules to form a connexon. Connexins are synthesized in the endoplasmic reticulum (ER) and oligomerize in the ER/Golgi or trans-Golgi network to form connexons. Connexons are subsequently transported to the plasma membrane by vesicular carriers travelling along microtubules. Connexons in adjoining cells fuse through disulfide bonding to form GJs (Figure 2.2). The combination of several connexins lead to diverse connexons
and GJ channels with different properties according to the needs of each cell type (Iossa et al., 2011).

**Figure 2.2** A diagram illustrating basic structures of connexins and undocked hemichannels present at the cell surface. Adopted from (Orellana et al., 2011).

Up to now, 21 connexins have been established in humans, each coding for a transmembrane protein with the same protein topology (Iossa et al., 2011). The crucial role of GJ-mediated intercellular communication (GJIC) for coordination of development, tissue function, and cell homeostasis is now well documented. In addition, recent findings have fueled the novel concepts that connexins, although redundant, have unique and specific functions, that GJIC may play a significant role in unstable, transient cell to cell contacts, and that GJ hemichannels by themselves may function in intra-/extracellular signaling (Segretain and Falk, 2004).

Gap junction biosynthesis and assembly are strictly regulated and intercellular junctions have a short half-life of only a few hours (Musil et al., 2000). Assembly of these channels is a complicated, highly regulated process that includes biosynthesis of the connexin subunit proteins on ER membranes, oligomerization of compatible subunits into hexameric hemichannels (connexons), delivery of the connexons to the plasma membrane, head-on docking of compatible connexons in the extracellular space at distinct locations, arrangement
of channels into dynamic, spatially and temporally organized GJ channel aggregates (so-called plaques), and coordinated removal of channels into the cytoplasm followed by their degradation (Segretain and Falk, 2004).

2.7 GJB2 (Cx26) structure

GJB2 gene is located on DFN1 locus on chromosome 13q12. GJB2 gene is also known as CX26, DFNA3, DFNA3A, DFNB1, DFNB1A, HID, KID, NSRD1, and PPK. The size of GJB2 is approximately 2.5 kb. It encodes GJB-2 protein, 26 kDa. It has about 226 amino acids (Kelsell et al., 1997). The structure of the GJB2 gene, as well the structure of other gap junction genes, is simple (Figure 2.3). An untranslated exon 1 is separated by an intron of 3179 bp length from exon 2, containing the uninterrupted coding region and the 3'-untranslated region (3'-UTR) (Iossa et al., 2011).

**Figure 2.3** GJB2 molecular structure and localization. GJB2 gene is localized in 13q11 chromosome and it is composed of two exons: only the exon 2 is a coding one, adopted from (Iossa et al., 2011).

2.8 Expression of Cx26 in inner ear

In the inner ear, Cx26 is expressed in the supporting cells, stria vascularis, basement membrane, limbus, and spiral prominence of the cochlea. The sensory hair cells of cochlea allow potassium ions to pass through during the mechanosensory transduction process of
normal hearing. These potassium ions are recycled across the supporting cells and fibrocytes at the base of hair cells through the gap junctions of the stria vascularis and back to the K+ rich endolymph (Figure 2.4). It is believed that mutations in the GJB2 gene would lead to complete or partial loss of function of the Cx26 protein, interfering with recycling of potassium ions and thus hampering the normal process of hearing (RamShankar et al., 2003).

![Figure 2.4 Schematic representation of the cochlea, showing the location of its different structures. The K+ recycling pathway is indicated. Adopted from (Jentsch, 2000).](image)

### 2.9 Heterogeneity of Genetic HL

About 300-500 genes are estimated to be necessary for hearing out of the 30,000-50,000 human genes. Genetic HL is mostly caused by a mutation in a single gene, but it represents extreme genetic heterogeneity. Genotype-to-phenotype correlation is important because different mutations in the same gene can lead to different phenotypes. The same mutation in a single gene can cause syndromic, non-syndromic, recessive or dominant hearing loss (Friedman and Griffith, 2003).

Despite this heterogeneity, mutations in the GJB2 gene are responsible for approximately 60% of NSARD, with a carrier frequency ranging between 2 and 5% depending on the ethnic group (Gasparini et al., 2000).
2.10 GJB2 mutations

Despite extraordinary genetic heterogeneity, mutations in one gene, GJB2, which encodes the Cx26 protein and is involved in inner ear homeostasis, are found in up to 50% of patients with NSARD. Because of the high frequency of GJB2 mutations, mutation analysis of this gene is available as a diagnostic test in many countries (Snoeckx et al., 2005).

The clinical consequence of certain mutations in the Cx26 gene is exclusively HL (non-syndromic deafness), whereas with other mutations the deafness is part of a syndrome (Marziano et al., 2003).

More than 100 recessive mutations of GJB2, nearly all affecting proper translation of the Cx26 protein, are currently known (Wilch et al., 2006; Bazazzadegan et al., 2011).

The most frequent recessive Cx26 mutation is a single base deletion 35delG that results in a frameshift at the 12th amino acid and premature termination of the protein (White, 2000). This mutation accounts for more than two-thirds of identified mutations (Schrijver, 2004). It still remains to be determined why this mutation has a relatively high frequency, but it has been suggested that 35delG is located in a hypermutable region. Both a local Chi consensus motif and a TGGGG sequence, which have been linked to β-globin gene mutations and to recombination in the immunoglobulin genes, could play a role. If slippage and mispairing of strands during DNA synthesis determine the high incidence of this mutation, however, ethnic background should not contribute much to variation in frequency. Yet, the prevalence of 35delG seems to markedly vary between populations (Denoyelle et al., 1997).

An alternative to the mutation hot-spot hypothesis has been offered by Van-Laer et al. (2001), who proposed that the high frequency of this variant results from a common founder. Because the mutation is thought to be evolutionarily ancient, haplotype sharing is observed in a small chromosomal region only. Although these hypotheses seem to be contradictory, both phenomena could have contributed to the high allele frequency of this single mutation (Van-Laer et al., 2001).

Other common recessive Cx26 mutations include nonsense mutations and small deletions/insertions (Table 2.2), most of which also lead to premature termination of
protein translation. Therefore, most of the frequently observed recessive mutations result in severely truncated connexin proteins that are unlikely to retain any channel activity and recessive HL may largely be a result of null mutations in Cx26 (White, 2000).

Genetic diagnosis is difficult because 10-50% of the affected persons have GJB2 mutations in only a single allele. In 50% of these patients, a deletion can be found which shortens the GJB6 gene. Digenic relation is now known to be an important cause of deafness in individuals, carrying a single mutation at the GJB2 locus along with a deletion at the functionally related GJB6 locus (Castillo et al., 2003).

Table 2.2 The most common recessive mutations of GJB2 gene.

<table>
<thead>
<tr>
<th>Name of mutation</th>
<th>Nucleotide change</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>35delG</td>
<td>Deletion of G at 35</td>
<td>Zelante et al., 1997</td>
</tr>
<tr>
<td>167delT</td>
<td>Deletion of T at 167</td>
<td>Bale et al., 1999</td>
</tr>
<tr>
<td>235delC</td>
<td>Deletion of C at 235</td>
<td>Fuse et al., 1999; Kudo et al., 2000</td>
</tr>
<tr>
<td>IVS1+1G&gt;A</td>
<td>G to A at –3172</td>
<td>Brownstein and Avraham, 2009</td>
</tr>
<tr>
<td>W77R</td>
<td>T to C at 229</td>
<td>Carrasquillo et al., 1997</td>
</tr>
<tr>
<td>W77X</td>
<td>G to A at 231</td>
<td>Kelsell et al., 1997</td>
</tr>
<tr>
<td>W24X</td>
<td>G to A at 71</td>
<td>Kelsell et al., 1997</td>
</tr>
<tr>
<td>W44X</td>
<td>G to A at 132</td>
<td>Green et al., 1999</td>
</tr>
<tr>
<td>E47X</td>
<td>G to T at 139</td>
<td>Denoyelle et al., 1999</td>
</tr>
<tr>
<td>G45E</td>
<td>G to A at 134</td>
<td>Fuse et al., 1999; Kudo et al., 2000</td>
</tr>
<tr>
<td>M34T</td>
<td>T to G at 101</td>
<td>Kelsell et al., 1997</td>
</tr>
<tr>
<td>M1V</td>
<td>A to G at 1</td>
<td>Estivill et al., 1998</td>
</tr>
<tr>
<td>Y65X</td>
<td>C to G at 195</td>
<td>Estivill et al., 1998</td>
</tr>
<tr>
<td>L79P</td>
<td>T to C at 236</td>
<td>Hamelmann et al., 2002</td>
</tr>
<tr>
<td>S85P</td>
<td>T to C at 253</td>
<td>Park et al., 2000</td>
</tr>
<tr>
<td>G12V</td>
<td>G to T at 35</td>
<td>Rabionet et al., 2000</td>
</tr>
<tr>
<td>L90P</td>
<td>T to C at 269</td>
<td>Denoyelle et al., 1999; Murgia et al., 1999</td>
</tr>
<tr>
<td>V95M</td>
<td>G to A at 283</td>
<td>Kelley et al., 1998</td>
</tr>
<tr>
<td>Q57X</td>
<td>C to T at 169</td>
<td>Wilcox et al., 1999</td>
</tr>
<tr>
<td>S19T</td>
<td>G to C at 56</td>
<td>Rabionet et al., 2000</td>
</tr>
</tbody>
</table>
2.11 Contribution of GJB2 (Cx26) gene to HL

Given the extraordinary genetic heterogeneity of NSHL, it was surprising to find that sequence variations at the GJB2 locus account for up to 50% of cases of NSARD in some populations. A recent model to explain this observation is based on the tradition of intermarriage between individuals with HL in some populations. A gradual increase in the proportion of HL due to a hypothetical autosomal recessive mutation would be a consequence of this assortative mating. More than 90 variants of the GJB2 gene have been reported, and many are rare. One variant generally predominates in any given population, such as 167delT in the Ashkenazi Jewish population, 35delG among Caucasians of northern European descent, 235delC in the Japanese population (Kenneson et al., 2002).

In Bethlehem-Palestine, 48 independently ascertained probands with NSARD were evaluated for mutations in the Cx26 gene. Of the 48 deaf probands, 11 (23%) were homozygous or compound heterozygous for mutations in GJB2. Five different mutations were identified: IVS1+1G>A, 35delG, 167delT, T229C, 235delC. The most common of these mutations are 35delG and 167delT (Shahin et al., 2002).

In Syria, a study performed to determine the prevalence of GJB2 gene mutations in a sample of forty-one patients with HL and direct DNA sequencing was used to analyze exon 2 of the GJB2 gene. Seven patients (17.07%) had at least one mutation. Five of these (71.43%) had the 35delG mutation, of which three were homozygous, whereas the other two cases were heterozygous. The other detected mutations were W77R in one case and 167delT with R184P mutations that together occurred as compound heterozygous in another case (Mahayri and Monem, 2012).

In Lebanon, 48 Lebanese pedigrees investigated with NSARD, 37 were Muslims, 10 Christians, and one Druze. Nearly 92% (44) of them were consanguineous. Sixteen of these families were linked to the DFNB1 locus (33%). In 15 of them, affected subjects were homozygous for the 30delG mutation in the GJB2 gene. In the last one, a non-consanguineous Christian Maronite family, affected subjects were compound heterozygotes for two novel mutations, a missense mutation, replacing arginine by histidine at codon 32 (R32H), and an insertion of an adenine in position 291 (291insA) creating a stop codon (Mustapha et al., 2001).
In Jordan, Sixty-eight unrelated Jordanian consanguineous families with at least two members having NSARD, were tested for mutations of the GJB2 gene by sequencing which revealed only the 35delG mutation, found at the homozygous state in 11 patients. It was not detected at the heterozygous state, and no other mutations of the gene were identified in any of the patients (Medlej-Hashim et al., 2002).

In the Mediterraneans, analysis of polymerase chain reaction (PCR) products from affected patients’ DNA showed two frameshift mutations in the Cx26 gene. Deletion of a G within a stretch of six Gs at position 35 of the GJB2 cDNA (mutation 35delG) leads to premature chain termination and is present in 63% of NSARD chromosomes. Deletion of a T at position 167 of GJB2 (mutation 167delT), also resulting in premature chain termination, was detected in another patient. In this study there were three patients heterozygous for mutation 35delG in whom there were no second mutation in the GJB2 coding region. In addition, there were 12 subjects with deafness linked to the DFNB1 locus who failed to show GJB2 mutations. These results suggest that mutations may be located in other regions of the connexin gene or that they may occur in neighboring genes mapping to chromosome 13q11-q12 which may also be involved in congenital deafness (Zelante et al., 1997).

In Egypt, ten patients from 10 different families with severe to profound HL were homozygous for the 35delG mutation. One patient of a family with NSARD had a heterozygous 35delG mutation combined with a heterozygous splice site mutation IVS1+1G>A. The 35delG mutation was the most common pathogenic mutation in this population (Snoeckx et al., 2005).

A study on 20 families, presently residing in an Israeli-Arab village in the lower Galilee, in whom both parents have normal hearing and ascertained through one child with a significant hearing defect. Genetic mapping by two-point and multi-point linkage analysis in 10 candidate regions identified the segregating gene to be on human chromosome 13q11 (DFNB1). Haplotype analysis, using eight microsatellite markers spanning 15 cM in 13q11, suggested the segregation of two different mutations in this kindred: W77R and 35delG and affected individuals were homozygotes for either haplotype or compound heterozygotes (Carraquillo et al., 1997).
In the Ashkenazi Jewish population, two frame-shift mutations in GJB2, 167delT and 30delG, were observed in the families with NSARD. The prevalence of heterozygosity for 167delT, which is rare in the general population, was 4.03%, and for 30delG the prevalence was 0.73%. The high frequency of carriers of mutations in GJB2 (4.76%) predicts a prevalence of 1 deaf person among 1765 people, which may account for the majority of cases of NSARD in the Ashkenazi Jewish population (Morell et al., 1998).

In Morocco, genomic DNA was isolated from 81 unrelated Moroccan familial cases with moderate to profound NSARD and 113 Moroccan control individuals. Molecular studies were performed using PCR-mediated site directed mutagenesis assay (PSDM), PCR and direct sequencing to screen for GJB2, 35delG and del(GJB6-D13S1830) mutations. GJB2 mutations were found in 43.20% of the deaf patients. Among these patients 35.80% were 35delG/35delG homozygous, 2.47% were 35delG/wt heterozygous, 3.70% were V37I/wt heterozygous, and 1 patient was E47X/35delG compound heterozygous. None of the patients with one or no GJB2 mutation displayed the common (GJB6-D13S1830) deletion. They found also that the carrier frequency of GJB2-35delG in the normal Moroccan population is 2.65% (Abidi et al., 2007).

In Tunisia, a study performed to determine the relative frequency of GJB2 allele variants on 138 patients with congenital HL belonging to 131 families originating from different parts of Tunisia. GJB2 mutations were found in 39% of families (51/131). The most common mutation was 35delG accounting for 35% of all cases (46/131). Four identified mutations in this study have not been reported in Tunisia; V37I, 235delC, G130A and the splice site mutation IVS1+1G>A (0.76%). These previously described mutations were detected only in families originating from Northern and not from other geographical regions in Tunisia (Riahi et al., 2013).

In Algeria, one hundred sixteen persons from fifty-eight families were tested by the method based on the principle of PSDM, followed by a BslI digestion. Mutation 35delG was diagnosed in sixteen families (11 homozygotes and 5 heterozygotes). The low allelic frequency (17.24%) and low ratio of individuals homozygous (13.8%) and heterozygous (6.9%) for the 35delG mutation suggest that there are other mutations in the GJB2 gene or other genes responsible for deafness in the Algerian population (Ammar-Khodja et al., 2007).
In the Kingdom of Saudi Arabia, consanguineous marriages are common practice and this is associated with a noticeably high frequency of inherited conditions affecting the resulting progeny, including NSARD. The population covered in this study is likely to have a multiethnic background caused by decades of religious and economic migration to this region. A total of 12 reported \textit{GJB2} mutations were identified in 17 out of 109 (15.59\%) NSARD cases. Biallelic \textit{GJB2} mutations were identified in 11 out of the 109 NSARD cases (10.09\%), with 35delG being the most common (7/11, 63.63\%). The remaining six patients were found to have monoallelic \textit{GJB2} mutations (Al-Qahtani et al., 2010).

A study performed on Kuwaiti patients to determine \textit{Cx26} gene mutations. Gene profiling and sequencing were performed to detect the presence and nature of \textit{Cx26} mutation. Of the 100 patients, mutation of \textit{Cx26} gene was detected in 15 patients (15\%), 9 (60\%) of which were heterozygous and 6 (40\%) were homozygous. The remaining 85 patients were negative for mutation \textit{Cx26}. Of the 15 \textit{Cx26}-positive cases, 12 (80\%) resulted from 35delG mutation. Of the 9 heterozygous cases, 6 (66.6\%) were positive for 35delG while 3 (33.3\%) were positive for 71G>A mutation. All 6 homozygous cases were positive for 35delG mutation (Al-Sebeih et al., 2014).

A study performed to investigate the prevalence of mutations in the \textit{Cx26} (\textit{GJB2}) gene in Omani population using both PCR-Restriction fragment length polymorphism (PCR-RFLP) and direct DNA sequencing methods. Two common \textit{GJB2} gene mutations (35delG and 167delT) were screened in 280 healthy controls and 95 deaf patients using two different PCR-RFLP methods. To investigate other \textit{GJB2} mutations, they have amplified and sequenced DNA from 51 unrelated deaf patients and 17 control subjects. None of the samples studied, either by RFLP or sequencing, revealed any deafness associated mutations in the coding region of the \textit{GJB2} gene. These findings disagree with many reports on the \textit{GJB2} gene, describing various mutations as the cause of congenital recessive deafness (Simsek et al., 2001).

In Iran, a study performed to investigate the prevalence of the \textit{GJB2} gene mutations using direct sequencing in 43 presumed NSARD subjects from 34 families in an Iranian population. Eleven different genetic variants were identified. \textit{GJB2}-related deafness mutations (35delG, 235delC, W24X, R184P and IVS1+1G>A) were found in 9 of 34 families (26.5\%). The 35delG was the most common mutation found in 5 of 34 families
(14.7%). One novel variant (–3517G>A) was found in the upstream region of the gene (Hamelmann et al., 2002).

Another study in Iran performed to investigate the contribution of GJB2 to the NSARD in the Iranian population. One hundred sixty eight persons from 83 families were studied. GJB2-related deafness was diagnosed in 9 families (4, 35delG homozygotes; 3, 35delG compound heterozygotes; 1, W24X homozygote; and 1, non-35delG compound heterozygote). The carrier frequency of the 35delG allele in this population was ~1% (1/83). Because the relative frequency of Cx26 mutations is much less than in the other populations, it is possible that mutations in other genes play a major role in NSARD in Iran (Najmabadi et al., 2002).

In China, DNA specimens collected from 3004 patients with NSARD from 26 regions of China were screened for the 235delC mutation. The coding exon of the GJB2 gene was PCR amplified, followed by restriction enzyme digestion with Apal and analysis by agarose gel. They showed that overall, 488 patients (16.3%) were determined to carry at least one 235delC mutant allele, with 233 (7.8%) homozygotes and 255 (8.5%) heterozygotes. Therefore, within the subpopulations examined, the frequency varies from 0% to 14.7% for 235delC homozygotes and from 1.7% to 16.1% for heterozygotes. On the basis of this survey of the patient cohort as stated, Chinese patients with NSARD appear to have a relatively higher 235delC frequency than that of other Asian populations (Dai et al., 2007).

In Japan, a study performed to analyze mutation for GJB2 in Japanese NSHL patients compatible with recessive inheritance. It was confirmed that GJB2 mutations are an important cause of HL in this population, with three mutations, 235delC, Y136X, and R143W, were especially frequent. Of these three mutations, 235delC was most prevalent at 73%. Surprisingly, the 35delG mutation, which is the most common GJB2 mutation in white subjects, was not found in this study (Abe et al., 2000).

In Italy and Spain, DNA samples from 82 families with NSARD and from 54 unrelated participants with apparently sporadic congenital deafness were obtained and the coding region of the GJB2 gene was analyzed for mutations. Also 280 unrelated people from the general populations of Italy and Spain were tested for the frameshift mutation 35delG. Forty-nine percent of participants with recessive deafness and thirty-seven percent
of sporadic cases had mutations in the \textit{GJB2} gene. The 35delG mutation accounted for 85\% of \textit{GJB2} mutations, six other mutations accounted for 6\% of alleles, and no changes in the coding region of \textit{GJB2} were detected in 9\% of \textit{DFNB1} alleles (Estivill et al., 1998).

A study on one 174 unrelated patients with prelingual NSARD to assess the significance of a mutation, IVS1+1G>A, to the NSARD among Iranian Azeri Turkish patients. Thirty nine patients had only one identified mutated allele, whereas hundred and thirty five patients were negative for coding region of \textit{GJB2}. All these patients were screened for IVS1+1G>A by applying PCR-RFLP assay. Among studied patients, nine compound heterozygotes with 35delG, 120delE, 235delC were identified. Additionally, six patients were detected with only one IVS1+1G>A mutated allele. In these patients, the other mutated allele was left unidentified. One patient was identified to be homozygous for IVS1+1G>A. Further studies carried out on parents of positive cases, showed that one of the healthy parents (mother) to be homozygous for IVS1+1G>A mutation. By self-report, this person had no HL, although it is possible that she has mild or moderate HL, which she is unable to detect. Her child was compound heterozygous (IVS1+1G>A/35delG) with profound deafness (Bonyadi et al., 2011).

A study performed on 60 patients from mostly large Turkish families with NSARD. \textit{GJB2} mutations were found in 31.7\% of the families, and the \textit{GJB2}-35delG mutation accounted for 73.6\% of all \textit{GJB2} mutations. The carrier frequency of \textit{GJB2}-35delG in the normal Turkish population was found to be 1.17\% (five in 429). In addition to the described W24X, 233delC, 120delE and R127H mutations, they also identified a novel mutation, Q80R, in the \textit{GJB2} gene (Uyguner et al., 2003).

In Pakistan, the Pakistani population has become an important resource for research on NSARD due to the availability of large extended and highly consanguineous pedigrees. Here is presented the first report on the prevalence of \textit{GJB2} variants in Pakistan. One hundred and ninety-six unrelated Pakistani families with NSARD were recruited for a study on the genetics of NSARD. DNA sequencing of the \textit{GJB2} coding region was done on two affected individuals per family. Homozygous putatively functional \textit{GJB2} variants were identified in 6.1\% of families. None of the putatively functional \textit{GJB2} variants were observed in the compound heterozygous state. The six putatively causative variants noted were W77X, W24X, 167delT, R32H, 120delE, and L90P, with W77X and W24X being the most common (Santos et al., 2005).
In Korea, a study performed to estimate the carrier frequencies of three GJB2 mutations, including 235delC, V37I, and G45E, in the general Korean population. A standard questionnaire of self-reported HL was used to identify and recruit subjects. Screening for three mutations was performed using an allele-specific PCR, PCR-RFLP, and direct DNA sequencing. A total of 1256 unrelated healthy individuals were analyzed in the present study. Of the 1256 individuals, 24 had GJB2 mutations; 11 were found to be heterozygous for 235delC, 11 were heterozygous and one was homozygous for V37I, and one was heterozygous for G45E. One individual had a compound heterozygous mutation of 235delC/V37I. The allele frequencies of 235delC, V37I and G45E mutations were 0.44%, 0.52% and 0.04%, respectively (Kim et al., 2011).

In Indonesia, forty patients with NSARD were studied to detect the common frameshift mutation (235delC) of Cx26 gene by using the PCR-RFLP analysis. But the results reflect no 235delC mutation in these patients. The possible explanation was that 235delC variant is not a cause of NSARD in Indonesia (Gaffar et al., 2009).

In Taiwan, a study performed to determine the mutation spectrum of the Cx26 gene among 324 Taiwanese patients with prelingual deafness and the carrier rate of gene mutation in another 432 unrelated control subjects. The coding region of the Cx26 gene was sequenced in both directions to detect mutation in all 756 samples. Among the 756 samples tested, 21 Cx26 variants were detected, including 7 novel ones. The 235delC mutation was the most common, accounting for 57.6% of the mutant alleles. Among patients, 48 (14.8%) had Cx26 gene mutations. In the control group, the carrier rate of Cx26 mutation was estimated at 2.8% (Hwa et al., 2003).
Chapter 3
Materials and Methods

3.1 Materials

3.1.1 Chemicals and Reagents

Chemicals and reagents used in this study are shown in Table 3.1. All chemicals were of analytical and molecular biology grade.

Table 3.1 Chemicals and reagents used in this study.

<table>
<thead>
<tr>
<th>#</th>
<th>Reagent</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Wizard ® Genomic DNA Purification Kit</td>
<td>Promega (Madison, USA)</td>
</tr>
<tr>
<td>2.</td>
<td>PCR Go Taq® Green Master Mix</td>
<td>Promega (Madison, USA)</td>
</tr>
<tr>
<td>3.</td>
<td>Agarose</td>
<td>Promega (Madison, USA)</td>
</tr>
<tr>
<td>4.</td>
<td>PCR primers</td>
<td>Promega (Madison, USA)</td>
</tr>
<tr>
<td>5.</td>
<td>Nuclease free water</td>
<td>Promega (Madison, USA)</td>
</tr>
<tr>
<td>6.</td>
<td>Ethidium bromide</td>
<td>Promega (Madison, USA)</td>
</tr>
<tr>
<td>7.</td>
<td>Ethanol 70%</td>
<td>(Sigma USA)</td>
</tr>
<tr>
<td>8.</td>
<td>Absolute Isopropanol</td>
<td>(Sigma USA)</td>
</tr>
</tbody>
</table>

3.1.2 Instruments and Disposables

The important instruments and disposables used in the present study are listed in Table 3.2.

3.2 Methodology

3.2.1 Study Design

The present study is a descriptive analytical design.

3.2.2 Study Location

Study subjects were selected from three Gaza Strip HL institutions and centers/societies (Mustafa Sadiq Rafii Secondary School for the Deaf, Atfaluna Society for the Deaf Children, Basma Center for Audiology & Speech Therapy). Experimental work was carried out at the Genetic Diagnosis lab of the Islamic University of Gaza.
Table 3.2 Instruments and disposables used in this study.

<table>
<thead>
<tr>
<th>#</th>
<th>Instrument</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Thermal Cycler</td>
<td>Eppendorf, Germany</td>
</tr>
<tr>
<td>2</td>
<td>Electrophoresis chambers and tanks (horizontal)</td>
<td>BioRad, USA</td>
</tr>
<tr>
<td>3</td>
<td>Electrophoresis power supply</td>
<td>BioRad, USA</td>
</tr>
<tr>
<td>4</td>
<td>Microcentrifuge</td>
<td>Sanyo, UK</td>
</tr>
<tr>
<td>5</td>
<td>Microwave Oven</td>
<td>L.G, Korea</td>
</tr>
<tr>
<td>6</td>
<td>Digital balance</td>
<td>AE adam, USA</td>
</tr>
<tr>
<td>7</td>
<td>Freezer, refrigerator</td>
<td>ORSO, pharml-spain</td>
</tr>
<tr>
<td>8</td>
<td>Micropipettes (0.1-2.5 μl / 0.5-10 μl / 5-50 μl / 20-200 μl / 100-1000 μl)</td>
<td>Dragon-lab, USA</td>
</tr>
<tr>
<td>9</td>
<td>Safety cabinet</td>
<td>N-Biotek, Inc</td>
</tr>
<tr>
<td>10</td>
<td>Gel documentation system</td>
<td>Vision, Scie-Plas Ltd, UK</td>
</tr>
<tr>
<td>11</td>
<td>Microfuge tubes for PCR-thin wall 0.2 ml capacity</td>
<td>Labcon, USA</td>
</tr>
<tr>
<td>12</td>
<td>Microfuge tubes-1.5 ml capacity</td>
<td>Labcon, USA</td>
</tr>
<tr>
<td>13</td>
<td>EDTA tubes</td>
<td>Hy. Labs. Israel</td>
</tr>
<tr>
<td>14</td>
<td>Disposable tips</td>
<td>Labcon, USA</td>
</tr>
</tbody>
</table>

3.2.3 Study population

The study population consisted of 70 deaf patients living in different regions of Gaza strip. The participants were from both genders and their age ranged from 2-27 years old. Apart from their deafness the subjects did not have any other serious health problems.

3.2.4 Ethical Considerations

The study protocol was presented for approval to the Ministry of Education, Directorate of Education-West of Gaza and to the Administrations of the three centers where the patients were recruited from. Approval and consent of parents of patients was also taken (Annex 2).

3.2.5 Instruments of the study

a. Questionnaire

b. Blood sample
3.2.6 Blood sample collection

Peripheral blood samples (~2 ml) were collected in EDTA tubes from the 70 unrelated patients diagnosed as having NSARD.

3.2.7 Exclusion criteria

In order to ensure that HL in the study subjects is most probably of the autosomal recessive non-syndromic type, a questionnaire was prepared for each patient to help exclude other HL causes as listed in (Annex 3) such as:

- Any event that preceded the deafness as swimming, showering, noise exposure, long term drug use (e.g. aminoglycosides) or trauma.
- Any recent or past episode of disease or infection as viral infection, meningitis, diabetes and others.
- Any event during mother’s pregnancy such as drugs intake, accident, infection, surgery or exposure to anesthesia.
- Any event during mother’s delivery such as premature delivery, low birth weight infant, lack of oxygen during delivery or difficulty during delivery.
- Some types of birth control method that the family uses such as oral contraceptives.

3.2.8 Extraction of DNA

Genomic DNA was prepared from peripheral blood lymphocytes using Wizard Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's protocol, and stored at -20°C until use. Extracted DNA was used in screening for the selected mutations.

3.2.9 Analysis of mutations by PCR

Specific primer sequences listed in (Table 3.3) were used to amplify the fragments containing the mutation. Then PCR-RFLP was performed for detection all five mutations, amplification was carried out in a thermocycler as listed in (Table 3.4), and In each PCR reaction a negative control, in which DNA wasn’t added, was used to confirm the absence of contaminants in the reagents. Finally, PCR products were digested using the specific restriction enzymes.
3.2.10 Electrophoresis

PCR products were analyzed by electrophoresis in a 1.5% agarose gel containing ethidium bromide, and the results were documented using a gel documentation system.

3.2.11 Selected mutations

In this study, samples were first screened for 35delG and 167delT mutations, when no 35delG or 167delT mutations was detected in deaf patients, screening was continued for the 235delC, IVS1+1G>A and W77R mutations.

We selected these mutations because population in Gaza strip is an extension of populations of neighboring Middle East countries (e.g., Lebanon, Syria and Jordan) and these mutations were reported as the most common in these countries.
Table 3.3 Primer sequences and the restriction enzymes used for detection of the different mutations in \textit{GJB2} gene.

<table>
<thead>
<tr>
<th>Restriction enzyme</th>
<th>Primer sequence (5’-3’)</th>
<th>Mutation</th>
<th>Size of the amplified fragment (bp)</th>
<th>Allele size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{BstI}</td>
<td>F: TCTTTTCCAGAGCAAAACGC R: GCTGGTGGAGTGTTTGTTCACACCCGC</td>
<td>35delG</td>
<td>89</td>
<td>Normal: 69+20</td>
<td>Cordeiro-Silva et al., 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hetero: 89+69</td>
<td>Homo: 89</td>
<td></td>
</tr>
<tr>
<td>\textit{MwoI}</td>
<td>F: GATTGGGGGCACGCTGCA R: CCCTTGATGAACCTCCTCTTCTTC</td>
<td>167delT</td>
<td>322</td>
<td>Normal: 322</td>
<td>Shahin et al., 2002</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hetero: 322+161</td>
<td>Homo:161</td>
<td></td>
</tr>
<tr>
<td>\textit{ApaI}</td>
<td>F: TGTGTGCATTCGTCTTTTCCAG R: GGTTGCCCTCATCCCTCTCAT</td>
<td>235delC</td>
<td>410</td>
<td>Normal: 262+148</td>
<td>Padma et al., 2009; Scott et al., 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hetero: 410+262+148</td>
<td>Homo: 410</td>
<td></td>
</tr>
<tr>
<td>\textit{HphI}</td>
<td>F: TCCGTAACCTTCCAGTCTCCGAGGGAGAG R: CCAAGGAGCTGTGGTGTCCAGCCGCC</td>
<td>IVS1+1G&gt;A</td>
<td>360</td>
<td>Normal: 242+118</td>
<td>Padma et al., 2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hetero: 360+242+118</td>
<td>Homo: 360</td>
<td></td>
</tr>
<tr>
<td>\textit{MspI}</td>
<td>F: CCATCTCCACATCCGGGCTC R: GCCTTGATGCGGACCTTCT</td>
<td>W77R</td>
<td>182</td>
<td>Normal: 100+82</td>
<td>Al-Achkar et al., 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hetero: 182+100+82</td>
<td>Homo: 182</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.4 Amplification protocol for detection the five mutations using thermal cycler.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Protocol</th>
<th>No. of cycles</th>
<th>Steps</th>
<th>Final Extension</th>
</tr>
</thead>
</table>
| 35delG   | 95°C for 5min | 35 | 95°C for 40sec  
65°C for 30sec  
72°C for 1min | 72°C for 2min |
| 167delT  | 94°C for 5min | 35 | 94°C for 15sec  
62°C for 15sec  
72°C for 30sec | 72°C for 5min |
| IVS1+1 G>A | 95°C for 5min | 32 | 95°C for 30sec  
59°C for 30sec  
72°C for 30sec | 72°C for 5min |
| 235delC  | 94°C for 5min | 35 | 94°C for 30sec  
60°C for 30sec  
72°C for 30sec | 72°C for 5min |
| W77R     | 94°C for 5min | 35 | 94°C for 30sec  
60°C for 30sec  
72°C for 30sec | 72°C for 5min |
Chapter 4
Results

4.1 Questionnaire analysis

Questionnaires were distributed in HL institutions and centers/societies in Gaza strip and were collected after being filled by the parents. Seventy patients were selected for this study who consisted of 37 (53%) males and 33 (47%) females. The age range of patients was 2-27 years, with an average age of 14.8 years. The patients were from different regions in Gaza strip as shown in (Table 4.1).

The results of the questionnaire revealed that consanguineous marriage is common between parents of patients (76%), and that there is a relevant family history in the family of the father (34%) or the mother (33%). Additionally, about 90% of the patients' parents have normal hearing with at least three children in their pedigree suffering from deafness without any other obvious abnormality. Collected data allowed us to consider the patients as having NSARD.

Table 4.1 Questionnaire results of the deaf patients.

<table>
<thead>
<tr>
<th>Questionnaire data</th>
<th>N</th>
<th>Percent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>37</td>
<td>53%</td>
</tr>
<tr>
<td>Female</td>
<td>33</td>
<td>47%</td>
</tr>
<tr>
<td>Educational level</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kindergarten and less</td>
<td>10</td>
<td>14.2%</td>
</tr>
<tr>
<td>Primary</td>
<td>16</td>
<td>22.8%</td>
</tr>
<tr>
<td>Preparatory</td>
<td>2</td>
<td>3%</td>
</tr>
<tr>
<td>Secondary</td>
<td>42</td>
<td>60%</td>
</tr>
<tr>
<td>Marital status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single</td>
<td>68</td>
<td>97%</td>
</tr>
<tr>
<td>Married</td>
<td>2</td>
<td>3%</td>
</tr>
<tr>
<td>Questionnaire data</td>
<td>N</td>
<td>Percent (%)</td>
</tr>
<tr>
<td>---------------------------------------</td>
<td>----</td>
<td>-------------</td>
</tr>
<tr>
<td>Geographical area</td>
<td></td>
<td></td>
</tr>
<tr>
<td>North</td>
<td>16</td>
<td>23%</td>
</tr>
<tr>
<td>Centre</td>
<td>18</td>
<td>26%</td>
</tr>
<tr>
<td>Gaza city</td>
<td>4</td>
<td>6%</td>
</tr>
<tr>
<td>South</td>
<td>32</td>
<td>45%</td>
</tr>
<tr>
<td>Consanguinity between the patient’s parents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>53</td>
<td>76%</td>
</tr>
<tr>
<td>No</td>
<td>17</td>
<td>24%</td>
</tr>
<tr>
<td>Hearing of the patient’s parents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deaf parents</td>
<td>7</td>
<td>10%</td>
</tr>
<tr>
<td>Non deaf parents</td>
<td>63</td>
<td>90%</td>
</tr>
<tr>
<td>Family history of deafness</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A relevant family history in the family of the father</td>
<td>24</td>
<td>34%</td>
</tr>
<tr>
<td>A relevant family history in the family of the mother</td>
<td>23</td>
<td>33%</td>
</tr>
</tbody>
</table>

### 4.2 GJB2 gene analysis

After the completion of the questionnaire, we employed a simple molecular test for the GJB2 gene mutations and it should be of considerable help to those patients to understand the cause of having the deafness.

Five different mutations in the GJB2 gene were screened: 35delG, 167delT, IVS1+1G>A, W77R and 235delC from seventy probands with NSARD. Screening for these five mutations in Cx26 was performed in two steps. First, the 35delG and 167delT mutations were screened in the deaf patients using restriction enzyme analysis of the PCR products. Second, when no 35delG or 167delT mutations were detected in deaf patients, screening was continued for the IVS1+1G>A, 235delC and W77R mutations.

Of the 70 deaf probands, 31 (44.3%) were homozygous or compound heterozygous for these five mutations, and there were 39 (55.7%) probands with NSARD who failed to show any of these mutations. This analysis started by screening 35delG and 167delT mutations in probands, since they represent the most frequent mutations in Cx26 gene in a previously reported Palestinian population from Bethlehem (Shahin et al., 2002).
But in our study, the results reveal no 167delT or W77R mutation and the most frequent of these five mutation was 35delG as indicated in (Table 4.2).

### 4.3 Allele frequency of the detected mutations among deaf patients

Total allele frequency = \( \frac{\text{Total mutant alleles}}{\text{Total number of alleles}} \)

\[
= \frac{(24 \times 2 + 1 \times 2 + 6)(70 \times 2)}{}
\]

= 0.4%

(Table 4.2) illustrates the allele frequency of the detected mutations among the deaf patients in this study. The results show that 35delG has the highest allele frequency.

#### Table 4.2 Frequency of mutations in Cx26 in patients with NSARD in Gaza strip.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Genotype</th>
<th>Frequency No. (%)</th>
<th>Allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>35delG</td>
<td>35delG/35delG</td>
<td>24 (34.3%)</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>35delG/IVS1+1G&gt;A</td>
<td>1 (1.4%)</td>
<td></td>
</tr>
<tr>
<td>IVS1+1G&gt;A</td>
<td>IVS1+1G&gt;A/35delG</td>
<td>1 (1.4%)</td>
<td>0.007</td>
</tr>
<tr>
<td>235delC</td>
<td>235delC/unknown</td>
<td>6 (8.6%)</td>
<td>0.043</td>
</tr>
<tr>
<td>167delT</td>
<td>Not detected</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>W77R</td>
<td>Not detected</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>31(44.3%)</td>
<td></td>
<td>0.4</td>
</tr>
</tbody>
</table>

### 4.4 Screening for the 35delG mutation

35delG was the most common mutant allele in Cx26 in Gaza strip. After restriction analysis for 35delG mutation, 25 of the 70 probands (35.7%) were found to be either heterozygous (1.4%) or homozygous (34.3%) for this mutation. Heterozygosity was recorded in only one proband (proband no. 15) who is compound heterozygous for 35delG/IVS1+1G>A. (Figure 4.1) illustrates a photo of agarose gel for 35delG restriction analysis by BstI.
4.5 Screening for the 235delC mutation

The second most prevalent GJB2 mutation was detected only in heterozygous form (one mutant copy) in six probands (8.6%), with no second mutation in the GJB2 coding region. (Figure 4.2) illustrates a photo of agarose gel for 235delC restriction analysis by ApaI.

![Image of agarose gel for 235delC restriction analysis by ApaI.](image)

**Figure 4.2** A photo of ethidium bromide stained agarose gel for 235delC restriction analysis by ApaI, M= DNA size marker (50 bp ladder), lanes 1, 2, 4, 5, 6 indicate wild-type samples for 235delC with two bands of 262 bp and 148 bp size, lane 3 indicates a heterozygous sample for 235delC with three bands of 410 bp, 262 bp and 148 bp size.
4.6 Screening for the IVS1+1G>A mutation

In this study, only one proband (1.4%) who is compound heterozygous for this mutation with 35delG mutation was detected. (Figure 4.3) illustrates an agarose gel for IVS1+1G>A restriction analysis by *HphI*.

![Figure 4.3](image)

**Figure 4.3** A photo of ethidium bromide stained agarose gel for IVS1+1G>A restriction analysis by *HphI*, M= DNA size marker (50 bp ladder), lanes 1, 2, 4, 5, 6 indicate wild-type samples for IVS1+1G>A with two bands of 242 bp and 118 bp size, lane 3 indicate heterozygous sample for IVS1+1G>A with three bands of 360 bp, 242 bp and 118 bp size.

4.7 Screening for the 167delT mutation

In our study, none of the 70 samples showed the 167delT mutation, and this is indicated in the results of agarose gel which demonstrated that all samples were normal for this mutation and therefore were not digested by *MwoI* restriction enzyme as shown in (Figure 4.4).

![Figure 4.4](image)
**Figure 4.4** A photo of ethidium bromide stained agarose gel for 167delT restriction analysis by *MwoI*, M= DNA size marker (50 bp ladder), lanes from 1-6 indicate wild type samples for 167delT with one band of 322 bp size.

### 4.8 Screening for the W77R mutation

As with 167delT the study also could not detect any W77R mutation in the 70 investigated samples. This is indicated by agarose gel results which demonstrated that all samples were normal for this mutation and were not digested by *MspI* restriction enzyme (Figure 4.5).

**Figure 4.5** A photo of ethidium bromide stained agarose gel for W77R restriction analysis by *MspI*, M= DNA size marker (50 bp ladder), lanes from 1-6 indicate wild type samples for W77R with two bands of 100 bp and 82 bp size.
Chapter 5

Discussion

HL is a common and highly heterogeneous sensory disorder. Genetic causes are thought to be responsible for more than 60% of the cases in developed countries (Petit et al., 2001). In the majority of cases, NSHL is inherited in an autosomal recessive pattern (Friedman and Griffith, 2003).

The gene first identified in 1997 to be associated with NSARD was GJB2. The most common cause of congenital, recessively inherited, sensorineural NSHL (Wilch et al., 2010).

GJB2 gene codes for Cx26 which belongs to a family of transmembrane proteins with about 20 members in humans (Kelsell et al., 1997). Hexamers of connexins (connexons) are displayed in the plasma membrane. Docking of connexons on the surfaces of two adjacent cells results in the formation of intercellular gap junction channels (Willecke et al., 2002). Several different connexins, including Cx26, have been shown to participate in the complex gap junction networks of the cochlea (Forge and Wright, 2002; Cohen-Salmon et al., 2004). It has been postulated that these networks play a key role in potassium homeostasis, which is essential for the sound transduction mechanism (Kikuchi et al., 2000).

In this study, we screened five mutations in the GJB2 gene in families with NSARD in Gaza strip, and the selection of these five mutations relied on their common presence in the West Bank and in the Middle East.

Our results revealed that the GJB2 mutations were evident in 31 (44.3%) probands, this result is higher than most of the results reported in the neighboring countries and other ethnic groups as summarized in (Table 5.1). GJB2 mutations in Gaza strip NSARD population have the highest frequency among previous studies listed in this study especially in Palestine (Bethlehem) in which GJB2 mutations contributed to 23% of the NSARD (Shahin et al., 2002). But in some countries such as Oman, GJB2 mutations were ruled out from being a cause of hereditary NSHL making genetic counseling for HL a difficult task to accomplish (Simsek et al., 2001). Therefore, every
country should screen and document their common NSARD mutations in order to deliver appropriate diagnosis and counseling.

Table 5.1 Frequency of Cx26 mutations among deaf populations of different ethnic origin.

<table>
<thead>
<tr>
<th>Country</th>
<th>N</th>
<th>Frequency (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Korea</td>
<td>1256</td>
<td>1.9</td>
<td>Kim et al., 2011</td>
</tr>
<tr>
<td>Ashkenazi Jews</td>
<td>546</td>
<td>4.76</td>
<td>Morell et al., 1998</td>
</tr>
<tr>
<td>Pakistan</td>
<td>196</td>
<td>6.1</td>
<td>Santos et al., 2005</td>
</tr>
<tr>
<td>Iran</td>
<td>168</td>
<td>11.0</td>
<td>Najmabadi et al., 2002</td>
</tr>
<tr>
<td>Taiwan</td>
<td>756</td>
<td>14.8</td>
<td>Hwa et al., 2003</td>
</tr>
<tr>
<td>Kuwait</td>
<td>100</td>
<td>15</td>
<td>Al-Sebeih et al., 2014</td>
</tr>
<tr>
<td>Saudi Arabia</td>
<td>109</td>
<td>15.59</td>
<td>Al-Qahtani et al., 2010</td>
</tr>
<tr>
<td>Jordan</td>
<td>68</td>
<td>16.2</td>
<td>Medlej-Hashim et al., 2002</td>
</tr>
<tr>
<td>Syria</td>
<td>41</td>
<td>17.07</td>
<td>Mahayri and Monem, 2012</td>
</tr>
<tr>
<td>Egypt</td>
<td>111</td>
<td>19.8</td>
<td>Mustapha et al., 2001</td>
</tr>
<tr>
<td>Indonesia</td>
<td>120</td>
<td>20</td>
<td>Snoeckx et al., 2005</td>
</tr>
<tr>
<td>Algeria</td>
<td>116</td>
<td>20.7</td>
<td>Ammar-Khodja et al., 2007</td>
</tr>
<tr>
<td>Palestine (Bethlehem)</td>
<td>48</td>
<td>23.0</td>
<td>Shahin et al., 2002</td>
</tr>
<tr>
<td>Japan</td>
<td>53</td>
<td>26.4</td>
<td>Sugata et al., 2002</td>
</tr>
<tr>
<td>Turkey</td>
<td>60</td>
<td>31.7</td>
<td>Uyguner et al., 2003</td>
</tr>
<tr>
<td>Lebanon</td>
<td>48</td>
<td>33.3</td>
<td>Mustapha et al., 2001</td>
</tr>
<tr>
<td>Italy and Spain</td>
<td>54</td>
<td>37</td>
<td>Estivill et al., 1998</td>
</tr>
<tr>
<td>Israeli Arab</td>
<td>75</td>
<td>38.7</td>
<td>Sobe et al., 2000</td>
</tr>
<tr>
<td>Tunisia</td>
<td>138</td>
<td>39</td>
<td>Riahi et al., 2013</td>
</tr>
<tr>
<td>Morocco</td>
<td>81</td>
<td>43.20</td>
<td>Abidi et al., 2007</td>
</tr>
<tr>
<td>Gaza strip</td>
<td>70</td>
<td>44.3</td>
<td>Present study</td>
</tr>
</tbody>
</table>

One mutation of this gene, namely 35delG (also known as 30delG), accounts for up to 85% of NSARD in some populations (Kenneson et al., 2002). The mutation involves deletion in a stretch of six G nucleotides, which lies in codon 10, resulting in a frameshift; a glycine is converted to a valine at codon 12 and a stop codon is formed at codon 13 (Sobe et al., 2000). In fact, the 35delG mutation is thought to occur in a hot spot site for mutations (Denoyelle et al., 1997; Kelley et al., 1998; Morell et al., 1998).
In our study, three different GJB2 mutations were identified among the probands namely, 35delG, 235delC and IVS1+1G>A. And the most frequent, 35delG, which represented 80.5% of all mutations detected in this study, and accounted for 35.7%, which was found in either heterozygous (1.4%) or homozygous (34.3%) form. This result was higher than that previous reported in some Arab countries such as Jordan (13.6%) (Medlej-Hashim et al., 2002), Egypt (10.8%) (Snoeckx et al., 2005), Algeria (20.7%) (Ammar-Khodja et al., 2007) and Tunisia (35%) (Riahi et al., 2013) as listed in (Table 5.2).

Also, 35delG mutation in this study has higher frequency than reported in previous study in Palestine (Bethlehem) which account for 14% (Shahin et al., 2002).

Furthermore, 35delG mutation in Gaza strip has lower frequency than other Arab countries such as Morocco (35.80%) (Abidi et al., 2007), Saudi Arabia (63.63%) (Al-Qahtani et al., 2010), Syria (71.43%) (Mahayri and Monem, 2012) and Kuwait (80%) (Al-Sebeih et al., 2014), and in some Mediterranean countries such as Italy and Spain in which 35delG account for 85% (Estivill et al., 1998).

Although 35delG commonly found in the Caucasian and white populations, this mutation was not reported in Taiwanese (Wang et al., 2002), Korean (Kim et al., 2011) and Japanese populations (Abe et al., 2000). This indicates that among the founders of those Asian populations the GJB2 35delG mutation is rare. In the contrary, 235delC is the most highly reported mutation in those Asian populations as discussed below. This also illustrates an interesting widespread genetic phenomenon where the common mutations of the same disease are usually different in populations of distant ethnicities.

The second most frequent mutation detected was 235delC, which is a frameshift mutation due to deletion of a single cytosine at position 235 and was described for the first time in Japan (Yuan et al., 2010). Also, it represents the most frequent known mutation in Asian populations (Hwa et al., 2003; Ohtsuka et al., 2003; Park et al., 2003). In our study, this mutation was detected in only heterozygous form in 8.6% of NSARD probands. This frequency is lower than that reported in Chinese (16.3%) (Dai et al., 2007), Japanese (73%) (Abe et al., 2000) and Taiwanese (57.6%) (Hwa et al., 2003), where, this mutation represent the most frequent one in those populations. On the other hand, this result is higher than previous study performed in Palestine (Bethlehem)
**Table 5.2** Frequency of 35delG mutation among deaf populations of different ethnic origin.

<table>
<thead>
<tr>
<th>Country</th>
<th>Frequency (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ashkenazi Jews</td>
<td>0.73</td>
<td>Morell et al., 1998</td>
</tr>
<tr>
<td>Israel</td>
<td>2.5-4</td>
<td>Sobe et al., 2000</td>
</tr>
<tr>
<td>Egypt</td>
<td>10.8</td>
<td>Snoeckx et al., 2005</td>
</tr>
<tr>
<td>Jordan</td>
<td>13.6</td>
<td>Medlej-Hashim et al., 2002</td>
</tr>
<tr>
<td>Palestine (Bethlehem)</td>
<td>14</td>
<td>Shahin et al., 2002</td>
</tr>
<tr>
<td>Iran</td>
<td>14.3</td>
<td>Peyvandi et al., 2011</td>
</tr>
<tr>
<td>Algeria</td>
<td>20.7</td>
<td>Ammar-Khodja et al., 2007</td>
</tr>
<tr>
<td>Tunisia</td>
<td>35</td>
<td>Riahi et al., 2013</td>
</tr>
<tr>
<td>Gaza strip</td>
<td>35.7</td>
<td>Present study</td>
</tr>
<tr>
<td>Morocco</td>
<td>35.8</td>
<td>Abidi et al., 2007</td>
</tr>
<tr>
<td>Saudi Arabia</td>
<td>63.63</td>
<td>Al-Qahtani et al., 2010</td>
</tr>
<tr>
<td>Syria</td>
<td>71.43</td>
<td>Mahayri and Monem, 2012</td>
</tr>
<tr>
<td>Turkey</td>
<td>73.6</td>
<td>Uyguner et al., 2003</td>
</tr>
<tr>
<td>Kuwait</td>
<td>80</td>
<td>Al-Sebeih et al., 2014</td>
</tr>
<tr>
<td>Italy and Spain</td>
<td>85</td>
<td>Estivill et al., 1998</td>
</tr>
<tr>
<td>Lebanon</td>
<td>94</td>
<td>Mustapha et al., 2001</td>
</tr>
</tbody>
</table>

by Shahin et al. (2002), where their results revealed that only one proband (2.08%) had the 235delC in a compound heterozygous form with 167delT (Shahin et al., 2002).

This mutation, however, has not been detected in Indonesia (Gaffar et al., 2009) or Malaysia (Ruszymah et al., 2005). This indicates that 235delC does not contribute to GJB2-associated deafness in these populations.

The third detected mutation was IVS1+1G>A (also known as –3170G>A). This splice site mutation is predicted to disrupt splicing, yielding no detectable mRNA (Shahin et al., 2002). However, not all genetic laboratories routinely test for this mutation, which lies outside the coding region of the GJB2 gene (Yuan et al., 2010).

In our study, this mutation found only in one proband (1.4%), who is a compound heterozygous for IVS1+1G>A with 35delG. This result is the same as the one previously reported in Palestine (Bethlehem) (Shahin et al., 2002), Tunisia (Riahi et al.,
2013) and Egypt (Snoeckx et al., 2005). The frequency of this mutation is lower than in Iranian Azeri Turkish where in those populations it was recorded as 4.9% (Bonyadi et al., 2011).

Mutation 167delT is a frameshift mutation that involves deletion of a T at position 167 of GJB2 and results in premature chain termination (Zelante et al., 1997). This mutation seems specific to Israeli Ashkenazi and Palestinian populations (Shahin et al., 2002). Whereas in a previous study in Palestine, this mutation accounted for 8.3% and was detected in four probands (Shahin et al., 2002). In Ashkenazi Jews 167delT accounted for 4.03% (Morell et al., 1998). Also this mutation has been observed in some Arab countries such as Syria, where the mutation was encountered in heterozygous form in four probands (8%) and in compound heterozygous form in one family (2%) (Al-Achkar et al., 2011).

In our study, this mutation was not found in the seventy investigated patients indicating that 167delT mutation is quite rare in our population and its involvement in NSARD in Gaza strip is not tangible.

W77R (also known as 229T→C) is a missense mutation leading to substitution of arginine for tryptophan at codon 77 in the second transmembrane domain of GJB2 (Shahin et al., 2002). This allele has been previously reported in an Israeli Arab family (Carrasquillo et al., 1997), and in Palestine (Bethlehem) where it was evident in only one proband (2.1%) who was homozygous for this mutation (Shahin et al., 2002). Moreover, the mutation was reported in one Syrian patient (Mahayri and Monem, 2012). Many other previous studies reported the lack of W77R mutation in their patients suggesting that this mutation is low in most populations. In the present study, this mutation was not recorded indicating that W77R mutation is low and its significance in NSARD in Gaza strip is not remarkable.

Discrepancies between different studies in terms of the frequency and type of GJB2 mutations associated with NSARD could be due to several reasons including: sample size (higher sample size increases the chance of detecting rare or exceptionally low mutations), precision of genotyping method employed, selection criteria of the patients investigated, rate of consanguineous marriage, and population genetic structure in terms of the type of circulating founder GJB2 mutations.
In more than half of the examined patients none of the five screened $GJB2$ mutations could be detected. NSARD in those patients may be due to other $GJB2$ mutations not tested in this study or due to mutations in other NSARD loci.

In conclusion, there is a significant contribution of $GJB2$ mutations to congenital NSARD in the Palestinian population of Gaza strip. Screening for $GJB2$ mutations particularly, 35delG, 235delC and IVS1+1G>A should be offered to NSARD patients to confirm diagnosis of their congenital deafness, to deliver proper genetic counseling for the affected individuals and their families and to provide the patients the optimal management. Moreover, defining the mutation responsible for the disease and with the advent of PGD families with NSARD children can exploit this technique for having offspring free of this ailment.
Chapter 6
Conclusion and Recommendations

6.1 Conclusion

The present study focused on screening five mutations in GJB2 gene in families with NSARD in Gaza strip namely, 35delG, 167delT, 235delC, IVS1+1G>A and W77R. The results of this study can be summarized as follows:

- *GJB2* mutations contributed to 44.3% of NSARD in Gaza strip.
- Among *GJB2* mutations investigated in this study 35delG, 235delC and IVS1+1G>A were detected in the patients whereas 167delT and W77R were not observed and therefore their contribution to NSARD in Gaza strip is not significant.
- In Gaza strip, the 35delG mutation is, as in many populations, proved to be the most prevalent in NSARD patients. It is the predominant founder mutation in our population.
- The high level of homozygosity for 35delG observed in this study reflects could be due to consanguinity. The consanguinity rate in the present study population reached 76%.
- In the present study, 235delC allele was detected only in a heterozygous form. The second mutation may be another *GJB2* that was not screened for or less likely may be present in an entirely different locus.
- IVS1+1G>A was the third detected mutation in our patients and was evident in only one proband in compound heterozygous form with 35delG.
- 167delT and W77R mutations were not detected in our study indicating these mutations do not have a major contribution to NSARD in Gaza strip.
- About half of the patients did not show any of the five screened *GJB2* mutations.
- Our findings indicate that screening for three mutations, 35delG, 235delC and IVS1+1G>A would allow the early diagnosis of about half of the NSARD cases in Gaza strip.
6.2 Recommendations

- Confirming NSARD in patients before undertaking genetic analysis of *GJB2* gene.
- Starting the *GJB2* mutation analysis with 35delG, 235delC and IVS1+1G>A in NSARD patient genetic workup.
- Screening for other known Cx26 mutations when NSARD patients do not show the above mentioned mutations.
- Performing *GJB2* DNA sequence analysis for NSARD patients lacking known mutations.
- Investigating other loci (e.g., Cx30) associated with NSARD.
Chapter 7
References


Annex 1. Location of four of the screened mutations on the coding sequence of *GJB2*

```
ATGGATTGGGCAGCTGAGACGTCTGGGGGATGTAACA
AACACTCCACCAGCATTCGAGATCTGGGTCACTGCCCTCTCTTC
ATTTTTCGATTATGTATCTCTGCTCGTTGTTGCTGCAAAGGGTGGTG
GGGAGATGAGCAGGCGACTTTTGTCGCAACACCCTGAGCCA
GGCTGCAAAGAAGTGTGCTACGATCAGACTACCTCTCCCATCTCCCA

35delG

GGCTGCAAGAACGTGTGCACGTACGCTACCTCTCCCCATCTCCCA

167delT

CATCCGGCTAGGCCCCGGCACTGAGCTCTATTCTGCTCCAGCCCA
GCGCTCCTAGTGCCAGACTGCTGCTACGGCTACGGGAGAGTGAAGA
AGAAGAGGAAGTTCATCAAGGAGAGATAAGAGTGAATTTAAG
GACATCGAGAGATCGAACACAAGGAGGCTCCGACATGGAAGCT
CCCTGTGCTGGACCTACACAGGAGCATCTCTCTCCCCAGTCATC
TTCAAGCGCCCTTCATGATCGTCTCTCTATGATCGTCAGCACGG
CTCTCCATGCAGGGCTGGTGAAGTGAAGCCTGGCCTGGGATGTA
CCCAACACTGTGAGACCTTTTTGTCGCTCGGGGACAGGAGAGA
CTGTCTTCACAGTGTCTGACTGAGGAGATGATGAGTGTCTTGACT
CTGCTGAAAGTGCTCAATTTGTTATTGTCTTTGCTAATTAGATATTG
TCTGGGAAGTCAAAAAGCCAG

35delG

167delT

W77R

235delC
```
Annex 2. A copy of the consent form

الموضوع
طلب موافقة أولياء الأمور

أنا الباحثة: بدرية فؤاد السماك، ممتحنة بالجامعة الإسلامية بغزة في كلية الدراسات العليا: قسم التحاليل الطبية، أقوم بعمل دراسة بعنوان:

دراسة الطفرات المتعلقة في جين الكونكسين 26 المسؤولة عن الصمم الوراثي المنحني الغير مرتبط بمتلازمة في قطاع غزة.

وهي أول دراسة في هذا المجال يتم إجراؤها على الأشخاص الصم في قطاع غزة، والتي ستعود بالفائدة على العائلات التي فيها أشخاص مصابين بالصم، حيث تقوم هذه الدراسة بالكشف عن أنواع الطفرات التي تؤدي إلى فقد في وظيفة جين الكونكسين 26 الذي يلعب دور كبير في حاسة السمع.

ومن المعلوم أن الطفرات تختلف من إقليم لأخر والتي لم تحدد حتى الآن في إقليم قطاع غزة، وإن معرفة نوع الطفرة يمكننا من الكشف عنها في (المرحلة الجينية) مما يساعد في علاج الأشخاص المصابين بالصم والذي وفره لنا العلم الحديث في مراحل حياتهم الأولى.

ولإتمام هذه الدراسة يتطلب من عائلة المريض تعبئة الاستبيان بدقة والموافقة على سحب 2-3 مل من المريض، وأنا بدوري سوف أقوم بفحص هذه العينة بطرق جينية دقيقة ومتقدرة بعمل الزراعة في الجامعة الإسلامية بمساعدة البروفيسور فضل الشريف، وسيتم تزويده العائلات بنتائج الفحوصات المخبرية، مع العلم بأن نتائج الفحوصات والمعلومات التي سوف يتضمنها الاستبيان سوف تستخدم للغرض البحثي فقط ولن يتم نشرها بأي صورة من الصور.

ملاحظة: إذا وافق ولي الأمر:
1. فلنحو التوقيع أسفل الصفحة.
2. تعبئة الاستبانة المرفقة.

توقيع ولي الأمر:........................
Annex 3. A copy of the questionnaire

Questionnaire

Non-syndromic Autosomal Recessive Deafness

1. Date: ……\.....\2013

2. Patient name:…………………………………………. Age: ………

3. Address of contact guardian (Mobile No):……………………………………

4. Gender:  □ Male  □ Female

5. Educational Level:  □ Primary  □ Preparatory  □ Secondary  □ University

6. Marital status:  □ Single  □ Married

7. Geographical area: □ North  □ centre  □ Gaza city □ South

8. Number of patient's male sibs who are deaf:  ……………………………

9. Number of patient's female sibs who are deaf:………………………………

10. Number of patient's male sibs with normal hearing:…………………………

11. Number of patient’s female sibs with normal hearing:…………………………

12. Is there some relationship (Consanguinity) between the patient’s parents?
   □ No
   □ Yes, what is the kind of relationship?…………………

13. Do both parents have normal hearing?
   □ Yes
   □ No, any one of the parents is deaf? □ Mother □ Father □ Both
14. Is there a relevant family history in the family of the Father?
   - Yes
   - No

15. Is there a relevant family history in the family of the mother?
   - Yes
   - No

16. Has the deafness been present since birth?
   - Yes
   - No, how long have you had a problem with deafness: ………………….

17. Is there any event that preceded the deafness?
   - No
   - Yes, 
     - Swimming
     - Showering
     - Noise exposure
     - Long term drug use (e.g., Aminoglycosides)
     - Trauma
     - Others

18. Is there a recent or past episode of diseases or infection?
   - No
   - Yes, 
     - Viral infection:  
       - Mumps
       - Measles
       - Smallpox
       - Meningitis
       - Diabetes
     - Others…………………..

19. Is there any event during mother’s pregnancy?
   - No
   - Yes, 
     - Administration of drugs
     - Surgery or exposure to anesthesia
     - Accident
     - Infection
     - Others……………………..
20. Is there any event during mother’s delivery?
   □ No
   □ Yes, □ Premature delivery
         □ Low birth weight infant
         □ Lack of oxygen during delivery
         □ Difficulty during delivery
         □ Others………………………….. 

21. What birth control method does the family use?

........................................................................................................................................................................