A STUDY ON *GJB2* AND *GJB6* GENE MUTATIONS AMONG MALAYS WITH NON-SYNDROMIC HEARING LOSS

by

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DEDICATION

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LIST OF ABBREVIATIONS

^{o}C	: degree celcius
µl	: microlitre
µM	: micromolar
A_{260}/A_{280}	: ratio of 260 absorbance over 280 absorbance
bp	: base pair
Buffer BL	: Lysis Buffer
Buffer BW	: Wash Buffer I
Buffer TW	: Wash Buffer II
Buffer AE	: Elution Buffer
Buffer PB	: Bind Buffer
Buffer NW	: Wash Buffer
Buffer EB	: Elution Buffer
Cx	: connexin
ddH_2O	: deionized distilled water
dsDNA	: double strand DNA
DGGE	: denaturing gradient gel electrophoresis
dHPLC	: denaturing High Performance Liquid Chromatography
DNA	: deoxyribonucleic acid
dNTPs	: dinucleotide triphospate
EDTA	: ethylenediamine tetraacetic acid
e.g.	: example

GJB2	: Gap Junction Beta-2
GJB3	: Gap Junction Beta-3
GJB6	: Gap Junction Beta-6
LOH	: loss of heterozygosity
K^+	: potassium ion
$MgCl_2$	: magnesium chloride
min	: minute
ml	: mililitre
mM	: milimolar
Na^+	: sodium ion
NCBI	: National Center for Biotechnology Information
ng/µl	: nanogram per microliter
NSHL	: non-syndromic hearing loss
PBS	: Phosphate buffered saline
PCR	: Polymerase chain reaction
rpm	: round per minute
SNP	: single nucleotide polymorphism
SSCP	: single-strand conformation polymorphism
Taq	: *Thermophilus aquaticus*
TBE	: Tris Boric EDTA
U	: unit
UV	: ultra violet
V	: voltage

vs : versus

KAJIAN TERHADAP MUTASI GEN *GJB2* DAN *GJB6* DI KALANGAN PESAKIT MELAYU YANG MENGALAMI KECACATAN PENDENGARAN JENIS TIDAK SINDROMIK

ABSTRAK

Kecacatan pendengaran merupakan kecacatan deria yang paling kerap berlaku pada manusia. Lebih kurang satu daripada seribu bayi yang baru dilahirkan di seluruh dunia lahir dengan kecacatan pendengaran samada dengan darjah kecacatan pendengaran yang ringan atau tiada pendengaran langsung. Kecacatan ini boleh disebabkan oleh dua faktor iaitu faktor genetik dan persekitaran dengan lebih 50% daripada kecacatan tersebut disebabkan oleh faktor genetik. Telah terbukti bahawa terdapat pelbagai gen yang terlibat dalam penyakit kecacatan pendengaran tidak sindromik (NSHL) iaitu sejenis kecacatan pendengaran tanpa gejala lain dan gen tersebut dikaji dalam penyelidikan ini. Mutasi pada gen *GJB2* telah dikenal pasti sebagai gen utama yang terlibat dengan kecacatan pendengaran tidak sindromik (NSHL). Satu gen lain yang berkaitan iaitu gen *GJB6* yang terletak berhampiran dengan gen *GJB2* pada kromosom mungkin berkaitan dengan kecacatan pendengaran jenis NSHL. Tujuan kajian ini adalah untuk mengenalpasti mutasi pada dua gen tersebut dan perkaitannya dengan kecacatan pendengaran jenis NSHL. Sebanyak 91 sampel sel tisu pipi pesakit NSHL dan 91 sampel sel tisu pipi subjek kawalan di Kelantan diambil untuk kajian ini. Tindak balas rantaian polimerase (PCR) digunakan untuk menggandakan gen *GJB2*. Produk PCR gen *GJB2* seterusnya disaring untuk mutasi dengan menggunakan teknik DHPLC dan

mutasi yang berjaya dikesan kemudiannya disah tentukan dengan penjujukan DNA. Sebelas mutasi dan polimorfisma dikenal pasti pada 32 pesakit dan 37 subjek kawalan setelah sebanyak 182 pesakit NSHL dan kawalan disaring. Walaubagaimanapun, semua mutasi dan polimorfisma tidak menunjukkan hubungan statistik secara signifikan dengan NSHL dan darjah kecacatannnya. Bagi gen *GJB6*, pemotongan gen dikenal pasti dengan menggunakan teknik pelbagai PCR yang mana gen β-globin digunakan sebagai kawalan dalaman. Semua 182 pesakit dan kawalan telah dikenal pasti tidak mempunyai pemotongan gen *GJB6* walaupun di antara mereka mempunyai mutasi gen *GJB2* pada satu alel, dua alel atau tidak. Kami percaya bahawa pengambilan sampel yang lebih banyak dan menyaring seluruh gen *GJB2* dan *GJB6* serta gen lain yang berkaitan akan membantu mengenal pasti dan mengesahkan hubung kait antara mutasi dan polimorfisma dengan darjah kecacatan pendengaran.

A STUDY ON *GJB2* AND *GJB6* GENE MUTATIONS AMONG MALAYS WITH NON-SYNDROMIC HEARING LOSS

ABSTRACT

Hearing loss is the most common congenital sensory defects in human. About one in a thousand newborn in the world is born with the abnormality, which may vary from mild level of hearing loss to profound loss. This loss can be caused by two factors, genetic and environmental factors and more than 50% of the defect is due to genetic causes. It has been proven that multi genes are involved in non-syndromic hearing loss (NSHL), a type of hearing loss without other symptoms and covered in this study. Mutations in *GJB2* gene have been shown to be a major role for congenital NSHL. A related gene, *GJB6* which is located adjacent to *GJB2* might be related and associated with NSHL. The objectives of this study are to identify the mutations in the two genes and study the association with NSHL. A total of 91 buccal cell samples of NSHL patients and 91 normal volunteer buccal cells samples in Kelantan were taken for this study. Polymerase chain reaction (PCR) was used to amplify the coding region of *GJB2* gene. The PCR product of *GJB2* coding region was preceded with screening for mutations using denaturing High Performance Liquid Chromatography (dHPLC) and mutations detected were confirmed by DNA sequencing. Eleven sequence variations including mutations and polymorphisms were found in 32 patients and 37 control subjects after 182 NSHL patients and controls were screened. However, all the variations did not show any statistically significant association with NSHL and the severity. For *GJB6*

gene coding region, the deletion was identified by multiplex PCR assay whereby β-globin gene was used as internal control. All 182 patients and controls were found to have no deletion of *GJB6* coding region irrespective of whether they have genetic variation in *GJB2* or not. Consequently, it is believed that a larger sample size and screening all regions in *GJB2* and *GJB6* and other related genes are necessary to verify the possible association between the mutations and polymorphisms and the severity of hearing loss in patients.

CHAPTER 1

INTRODUCTION

1.1 Hearing loss

Hearing loss happens when someone could not hear voices and sounds due to problems with one or more parts of the ear or ears. It is also called hearing impairment or deafness. Since they are unable to hear their own voices when they do try to speak, they therefore cannot imitate themselves (Martin and Grover, 1986).

One in a thousand babies is born with hearing loss, making it the most common sensory type of birth defect (Watkin, 1996; Watkin and Baldwin, 1999). A study done by Abdullah *et al*., (2006) in Hospital Universiti Kebangsaan Malaysia (HUKM), Malaysia showed that the prevalence of hearing loss is 0.42%. Hearing loss can be due to environmental factors and genetic defects. Fifty one percent of hearing loss cases are caused by genetic factors and 77% are inherited in an autosomal recessive pattern (Morton, 1991). To date, it is believed that more than a hundred genes may be involved in hearing loss with several of these genes have been identified; two of them are gap junction protein beta-2 (*GJB2*) gene and gap junction protein beta-6 (*GJB6*) gene (Van Camp and Smith, 1999).

1.2 Hearing – How it works

Our ear is made up of three different sections; outer ear, middle ear and inner ear. Each section has a specific function. The outer ear collects sound waves and the middle ear converts the sound energy into a mechanical force, then it is transmitted to the inner ear thus converting it to electrical energy and sent via the auditory nerve to the brain (Freeland, 1989).

The outer ear comprises of pinna, which is shaped like a shell and its function is to pick up sound waves. The pinna leads to the ear-canal at the bottom of which the eardrum (tympanic membrane) is located. The ear drum is the division between the outer and middle ears (Freeland, 1989).

The middle ear is an air-containing space which contains three unusually shaped ossicles; the malleus (hammer), incus (anvil) and stapes (stirrup). The sound waves received by the eardrum will cause the vibration and this vibration will pass through the malleus, incus and stapes, thus converting sound energy into mechanical energy. This energy is directed on to a membrane (oval window) to which the stapes is connected and which is 22 times smaller than the size of the eardrum. The chain of ossicles thus acts as much as a hydraulic press; it magnifies 22-fold the small pressures on the surface of the eardrum and transmits them to the oval window, then sends the mechanical energy into the inner ear for conversion into electrical activity (Freeland, 1989).

The inner ear is an extraordinarily complicated mechanism, comprises a coiled structure called the cochlea. One end of the cochlea is connected to the oval window; the other end is also in contact with another part of the middle ear through another membrane called the round window. The inner ear filled with fluid and when the stapes moves, a ripple is sent right through the fluid of the inner ear to the round window. The movement of the inner ear fluid deflects a thin membrane in the centre of the cochlea called basilar membrane. Delicate cells like tiny hairs balance on this membrane (the organ of Corti) and connect directly to a multitude of nerve endings, which join together to form the auditory nerve (Freeland, 1989) (**Figure 1.1**).

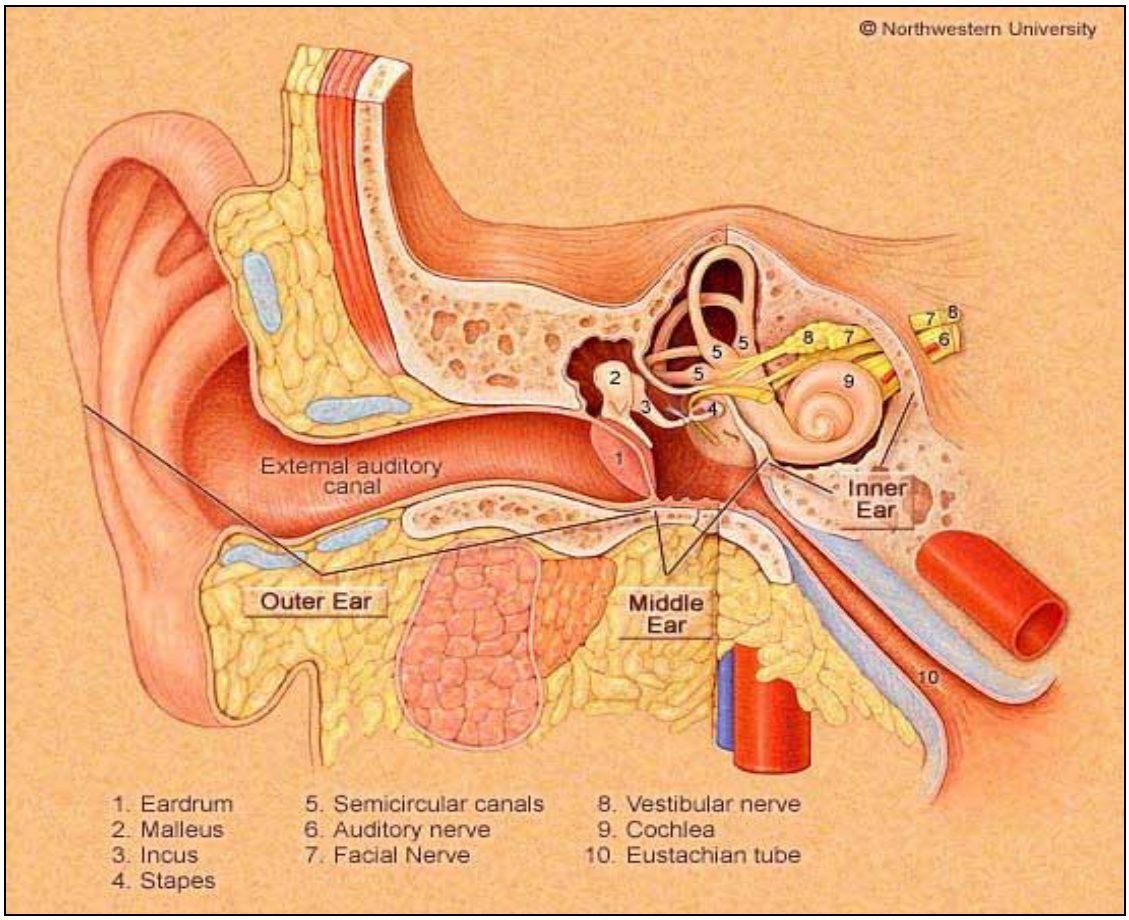


Figure 1.1: Cross-section of the outer, middle and inner human ear (http://www.dizziness-and-balance.com/disorders/hearing/hearing.html)

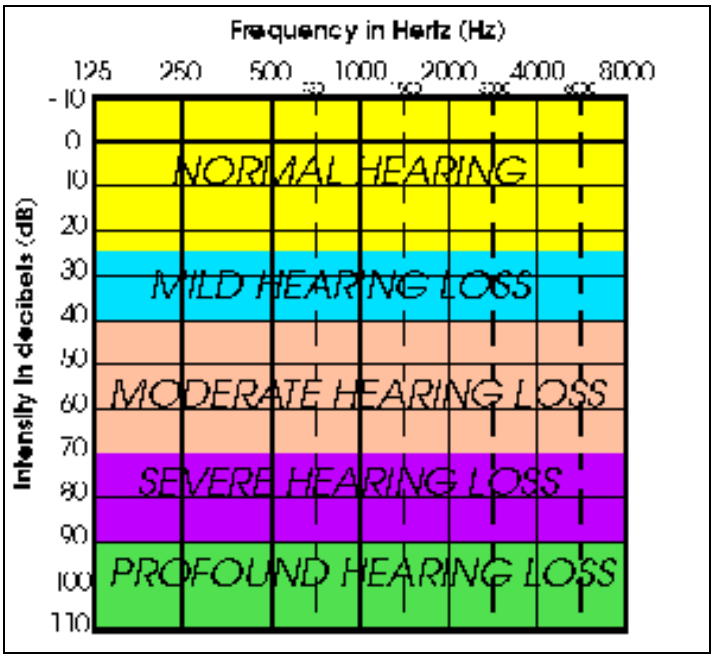
1.3 Degree of hearing loss

Hearing loss or deafness (and sound in general) is measured in decibels (dB). Hearing loss can be classified in different degrees according to the intensity: mild (30 to 45 dB), moderate (50 to 65 dB), severe (70 to 90 dB) and profound (>95 dB). Table 1.1 shows the relationship between the decibel hearing loss and degree of difficulty it may cause (Martin and Grover, 1986).

1.3.1 Pure Tone Audiometry (PTA)

Pure tone audiometry is pure tone test to determine the type and degree of hearing loss for both right and left ear. PTA is a behavioral test used to measure hearing sensitivity (Kurtz Jr, 1994) (**Figure 1.2**). A calibrated machine called an audiometer is used to present tones at different frequencies (pitches) and at different intensity (loudness) level. The responses from the emitted sounds are recorded and plotted on a graph called an audiogram. The frequency of the sound is referred to in Hertz (Hz) while the intensity is measured in decibels (dB). The PTA testing is done between frequency 125 and 8000 Hz. Once the audiogram is completed, the audiologist computes the pure tone average for each ear. It is the average of hearing thresholds at 500, 1000, and 2000 Hz, which are considered to be the major frequencies for speech. The pure-tone average represents the degree of hearing loss in decibels, not a percentage (http://www.asha.org/public/hearing/testing/assess.htm).

a



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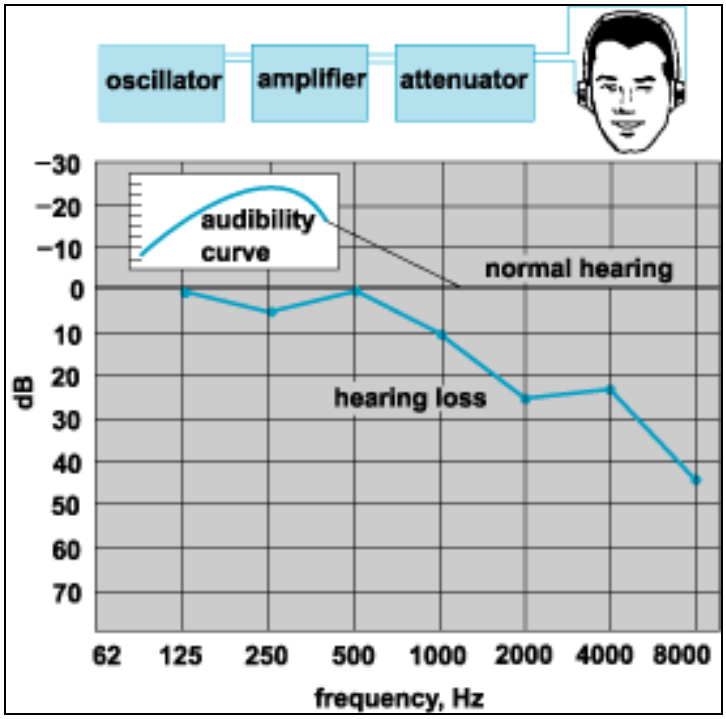


Figure 1.2: Examples of pure tone audiogram. (a) Scale of degrees of hearing level (http://www.springvalleyhearingctr.com/hearingassessment) (b) The audiogram in the illustration reveals a hearing loss for tones above 500 Hz (http://content.answers.com/main/content/img/McGrawHill/Encyclopedia/images/CE062100FG0010.gif)

1.4 Types of hearing loss

Classification of hearing loss is based on the localization of the point at which the auditory pathway has broken down. It determines whether the patients's hearing loss is conductive, sensorineural, central, and functional or a combination of these (Sataloff and Sataloff, 2005).

Conductive hearing loss is caused by any condition that interferes in the transmission of sound through the external and middle to the inner ear. If the damage is in the middle ear, it may involve the stapes, as in otosclerosis or the mobility of the eardrum and ossicles caused by fluid (Sataloff and Sataloff, 2005). Hearing tests on patient with conductive hearing loss show the inner ear or nerve function to be normal but air conduction to be reduced (Freeland, 1989). Most patients with this type of loss have a mild degree of loss, correctable and can be improved by medical treatment (i.e. surgical) (Ballantyne, 1993; Sataloff and Sataloff, 2005).

Sensorineural hearing loss happens when inner ear or auditory nerve or both is/are damaged. The term 'sensory' hearing loss is applied when the damage is localized in the inner ear while 'neural' implies the damage is in auditory nerve. The cochlea has ~30 000 hair cells that connect with nerve endings. The tiny hair cells could be damaged from a variety of causes. In the majority of cases, the loss is not curable (Sataloff and Sataloff, 2005).

In central hearing loss the damage is situated in central nervous system. The damage may be at any point from auditory nuclei in the medulla oblongata to the cortex. Formerly, central hearing loss was described as a type of ‘perceptive deafness’ (Sataloff and Sataloff, 2005).

Functional hearing loss is described when there is no detectable organic damage to auditory pathways but some underlying psychological or emotional problem is at fault (Sataloff and Sataloff, 2005).

Patients, who experienced two or more types of hearing impairment, are described as having mixed hearing loss. However, for practical purposes this term is used only when conductive hearing loss accompanied by a sensory or a neural (or a sensorineural) loss in same ear (Sataloff and Sataloff, 2005).

Table 1.1: The effect of the different degrees of hearing loss (Martin and Grover, 1986)

Decibels of hearing loss	Degree of impairment	Practical effect on hearing
Up to 25	Within normal range	Little effect
30 – 45	Mild	Difficulty with quiet voices
50 – 65	Moderate	Difficulty with many sounds
70 – 90	Severe	Cannot hear speech without a hearing aid
Over 95	Profound	Can hear only a little even with a powerful hearing aid

1.5 Non-syndromic hearing loss (NSHL)

Hearing loss is a symptom of many injuries and diseases. Therefore, it may expressed at any age and classified in many ways, such as genetic vs. acquired, syndromic vs. non-syndromic, prelingual vs. postlingual, and conductive vs. sensorineural (Morton, 1991).

Hearing loss can be due to environmental factors, genetic defects or a combination of these factors. Approximately 25% of children with hearing loss in the United States (U.S) are caused by environmental factors, such as prematurity, infections, exposure to ototoxic medications and trauma. It is estimated that at least 50% of prelingual hearing loss is caused by genetic changes, whereas the etiology remains obscure in the remaining 25%. Most of the cases, however, are assumed to be of genetic origin. Thus, genetic causes account for the largest proportion of all cases of prelingual hearing loss (Avraham, 2001).

Clinically, hearing loss may be associated with other disorders in the form of syndromic hearing loss or as an isolated finding, non-syndromic hearing loss. However, both syndromic and non-syndromic phenotypes can result from the mutation in the same gene (e.g *MYO7A, PDS*). Syndromic hearing loss tends to be less genetically heterogenous than non-syndromic, but more than one locus has been identified for several syndromes (Avraham, 2001; Keats and Berlin, 1999).

Hearing loss can follow a pattern of autosomal recessive, autosomal dominant, X-linked and mitochondrial inheritance. Allelic mutations in some genes can cause recessive and dominant hearing loss and recessive hearing loss may be caused by a combination of two mutations in different genes from the same functional group (Schrijver, 2004).

Autosomal dominant deafness loci are designated *DFNA*, autosomal recessive loci designated *DFNB* and X-linked loci, *DFN*. The loci are numbered according to the order in which they were mapped, *DFNA1* being the first autosomal gene mapped in 1992 (Van Camp and Smith, 1999).

Approximately 70% of genetic hearing loss is non-syndromic in nature. The largest proportion (about 80%) is inherited in an autosomal recessive mode, 18% is inherited in an autosomal dominant mode and 2% is X-linked. Mitochondrial/maternal inheritance also contributes to a small (1%) proportion of NSHL (Avraham, 2001) (**Table 1.2**).

Over 100 genes may be involved in non-syndromic hearing loss and the chromosomal location for over 80 have already been found. In the field of NSHL, 21 genes associated with autosomal recessive inheritance, 20 associated with autosomal dominant inheritance and one with X-linked recessive transmission have been identified and characterized (http://uia.ac.be/dnalab/hhh) with the most dramatic recent discovery is the high incidence of mutations found in the gap junction protein, connexin 26 (locus designation, *GJB2*) (Sobe *et al*., 2000).

Two additional genes have been implicated in hearing loss which are connexin 30 (*GJB6*) (Xia *et al*., 1998) and connexin 31 (*GJB3*) (Liu *et al*., 2000), both in non-syndromic hearing loss.

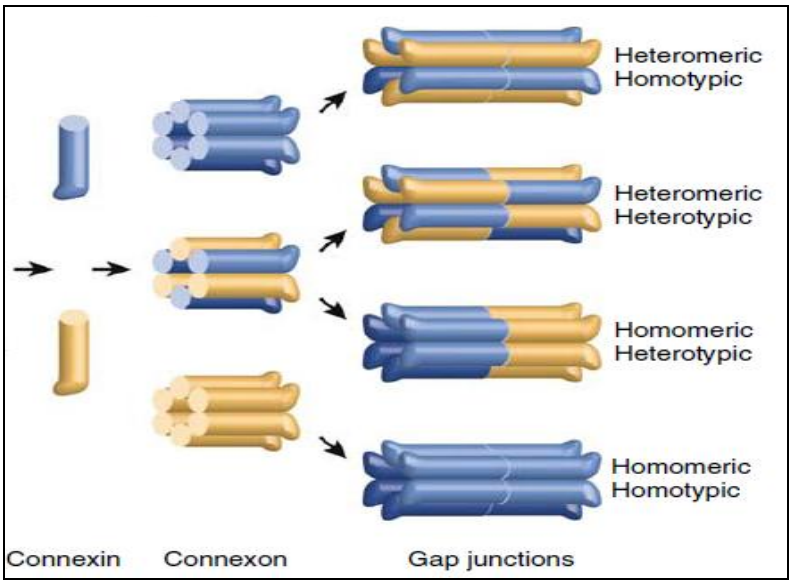
Table 1.2: Causes of hearing loss (Bitner-Glindzicz, 2002)

Genetic (Syndromic and non-syndromic)	**Environmental**
• Autosomal recessive • Autosomal dominant • X-linked • Mitochondrial	• Ototoxic medication • Severe neonatal jaundice • Head trauma • Noise exposure • Low birth weight • Infection: prenatal, e.g.: toxoplasmosis, rubella; postnatal, e.g. meningitis • Severe neonatal jaundice • Prematurity

1.6 Gap junction proteins in cochlea

The gap junction proteins, encodes connexins which is a component of connexons that allows molecules to pass from cell to cell. This organization requires the membranes of two neighboring cells to come close to each other (White and Bruzzone, 1996). Six connexins form a connexon, which then aligns in the extracellular space to complete the formation of gap junction channels. Two different connexins can interact with each other to form homomeric (all the connexin are same), heteromeric (two different connexins within a single connexon) and heterotypic (two different homomeric connexons) channels, which differ in their content and spatial arrangement of connexins subunits, while gap junction channels which are made from only one type of connexin is called homotypic channel (Sosinsky, 1995; Chang *et al*., 2003) (**Figure 1.3**).

a



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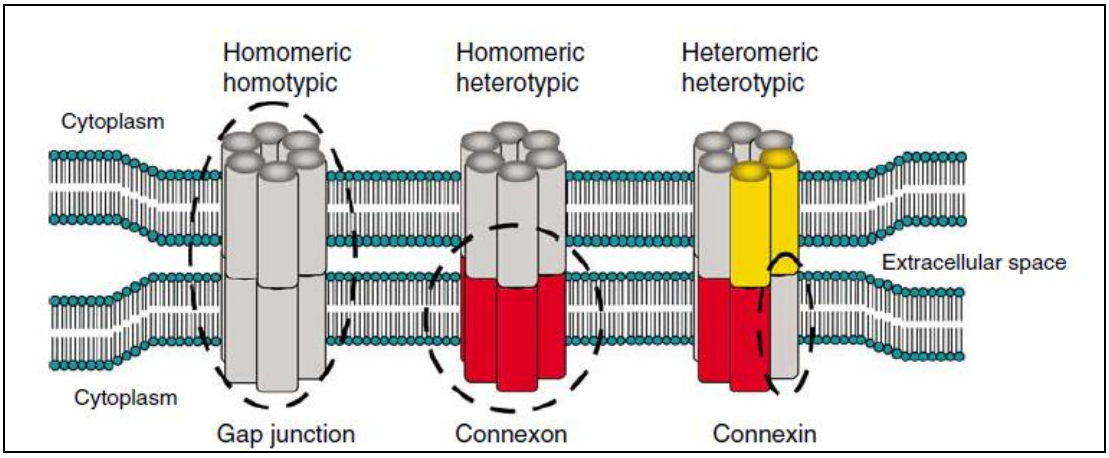
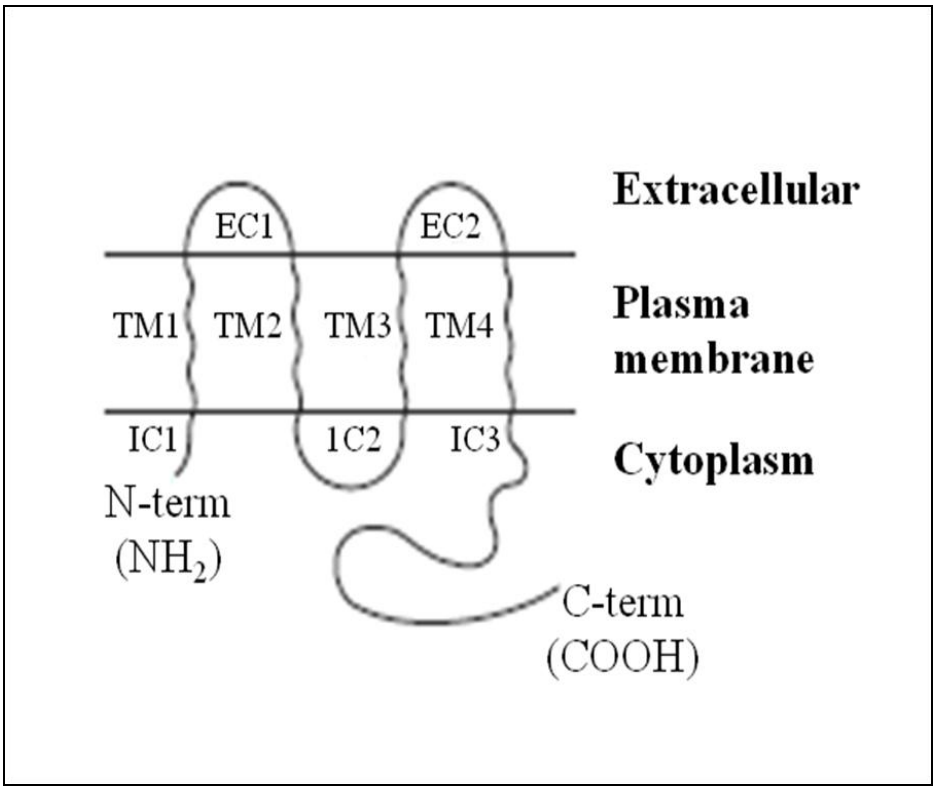


Figure 1.3: Schematic representation of connexins and gap junctions. **(a)** Blue and yellow connexins interact with each other to form homomeric, heteromeric and heterotypic channels, which differ in their content and spatial arrangement of connexins subunits. Adapted from Meşe *et al*., (2007) with modification **(b)** Six connexins form a connexon. Two connexons of neighbouring cells form pores; allow intercellular transport of small molecules (Wagner, 2008 - with modification)

Connexin family members share a similar structural topology. Each connexin has four transmembrane domains (TM1-TM4) that constitute the pore of the channels, with two intercellular loops (IC1 and IC2) and two extracellular loops (EC1 and EC2). Two terminal tails, N- (NH_2) and C- (COOH) termini are cytoplasmic. The length of the cytoplasmic C-terminus varies greatly among connexins and the cytoplasmic loop shows some variation in length. Otherwise the overall structure of the molecules is highly conserved (Bennett *et al*., 1991; Simon and Goodenough, 1998) **(Figure 1.4)**.

a



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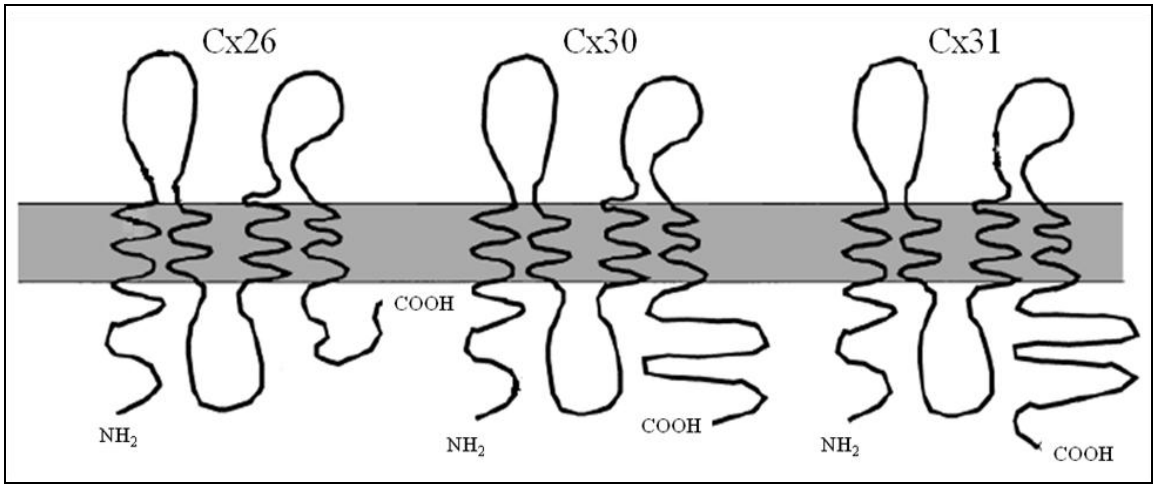

Figure 1.4: Schematic representation of basic connexin **(a)** Schematic representation of connexin. TM1-TM4; transmembrane domains, EC1-EC2; extracellular loops, IC1-IC3; intercellular loops and N- and C-termini; terminal domains. Adapted from Simon and Goodenough, 1998, with modification **(b)** Schematic representation of connexin 26, 30 and 31. The length of C-terminus show some variation, also the length of cytoplasmic loop. Other regions are highly conserved among connexins (Rabionet *et al*., 2000, with modification)

The amino terminus plays an important role in voltage gating, dynamically regulating gap junction properties by noncovalent or covalent modifications in the surrounding amino acids. The extracellular loops are critical for docking between two connexons and connexon compatibility. The transmembrane domains form the pore of the gap channel and it is important in channel permeability (Krutovskikh & Yamasaki, 2000).

Connexin 26 and 30, encoded by *GJB2* and *GJB6* gene, which are highly expressed in epithelial supporting cells of the mammalian cochlea, have been speculated to have a crucial role in the recycling of potassium ions (K^+) from the hair cells back to the endolymph of the cochlear duct. (Kikuchi *et al*., 1995; Petit *et al*., 2001).

Cochlea, a structure in the inner ear, contains transduction machinery to sense the vibration transmitted from the middle ear after sound stimulus (Martinez *et al.,* 2009) (**Figure 1.5**). Cochlea is formed by three adjacent and paralleled tubular compartments; scala media, scala tympani and scala vestibule. The epithelial cells, fibrocytes and receptor cells which are located in the wall of tubular compartments are the principal cellular components of cochlea. These compartments are filled with two types of fluid, endolymph and perilymph. The endolymph possesses a high concentration of potassium ion (K^+) and low level of sodium ion (Na^+) fills the scala media. The perilymph contains high concentration of Na^+ and low K^+ fills the scala vestibuli and scala tympani (Wangemann, 2006; Zhao *et al.,* 2006) (**Figure 1.6**).

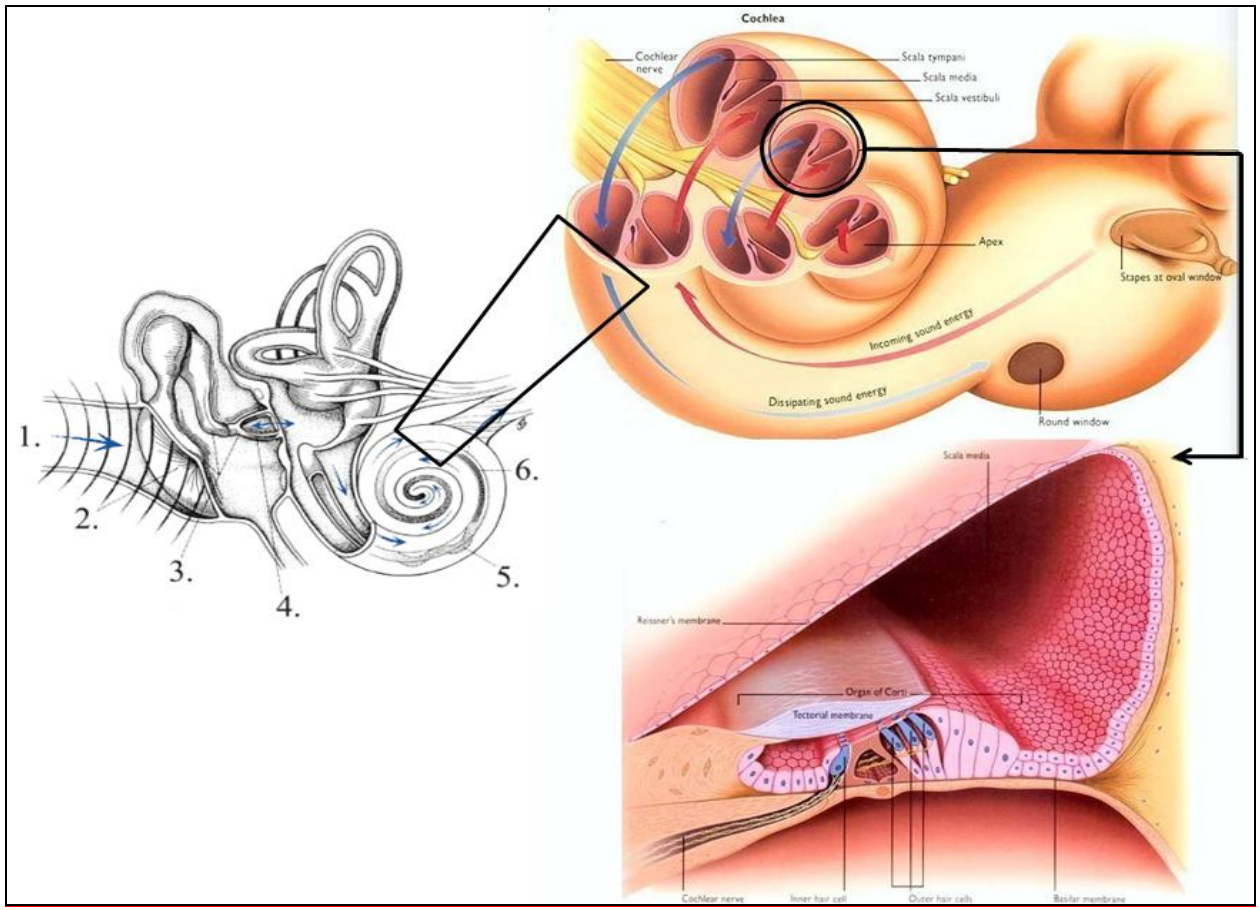

Figure 1.5: Flow diagram of human auditory pathway (http://eardoctors.org/med_info/images_med_info/earworks.gif and http://universe-review.ca/I10-85-cochlea.jpg)

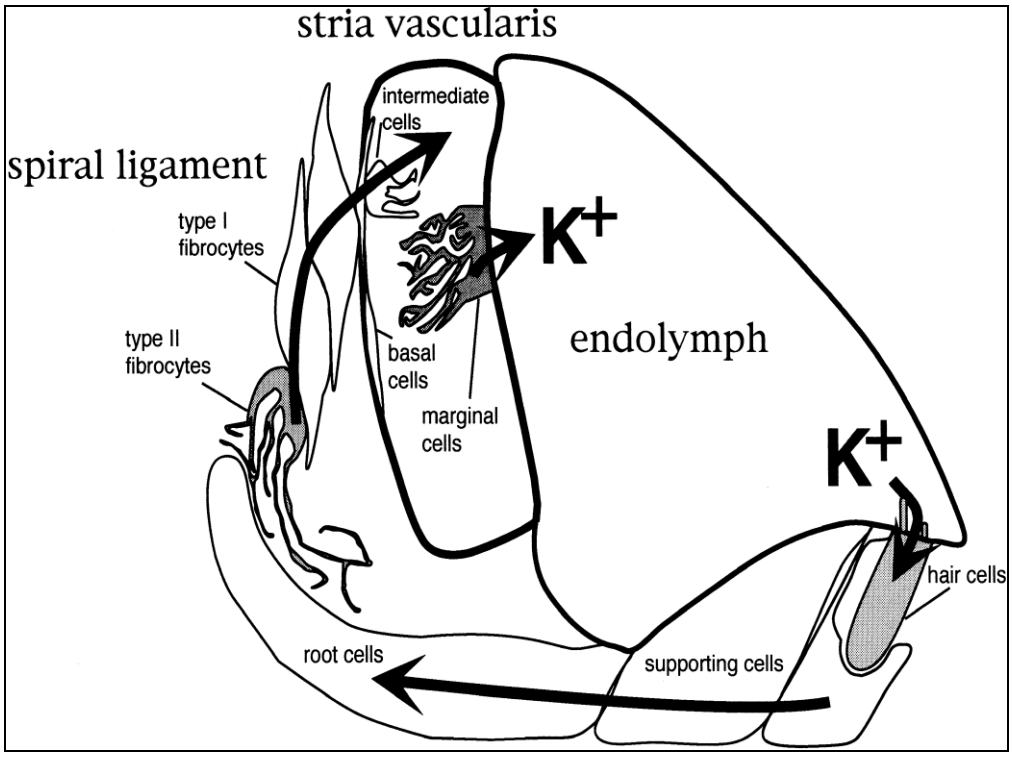

Figure 1.6: Schematic illustration indicating possible pathway for the transport of K^+ in the cochlea (Kikuchi *et al*., 2000)

In the mammalian cochlea, there are two independent gap junction systems, epithelial cell gap junction system and connective tissue cell gap junction system, which are necessary for the normal hearing function (Zhao *et al*., 2006). The first system, the epithelial cell gap junction system, is mainly composed of all organ of Corti supporting cells, and also includes interdental cells in the spiral limbus and root cells within the spiral ligament. The second system, the connective tissue cell gap junction system, consists of strial intermediate cells, strial basal cells, fibrocytes in the spiral ligament, mesenchymal cells lining the bony otic capsule facing the scala vestibuli, mesenchymal dark cells in the supralimbal zone, and fibrocytes in the spiral limbus (Kikuchi *et al*., 2000).

Activation of hair cells by acoustic stimuli induces influx of K^+ from the endolymph to sensory hair cells. This K^+ is released basolaterally to the extracellular space of the organ of Corti, from which they enter the cochlear supporting cells. Once inside the supporting cells, the ions move via the epithelial cell gap junction system laterally to the lower part of the spiral ligament. The K^+ is released into the extracellular space of the spiral ligament by root cells and taken up by type II fibrocytes. This uptake incorporates K^+ into the connective tissue gap junction system. Within the system, the K^+ passes through the tight junctional barrier of the stria vascularis and are released within the intrastrial extracellular space. The marginal cells of the stria vascularis then take up K^+ and return it to the endolymphatic space, where it can be used again in sensory transduction (Kikuchi *et al*., 2000).

It is highly probable that mutations of connexin genes cause dysfunction of cochlear gap junctions and thereby interrupt K^+ recirculation pathway (Kikuchi *et al*., 2000). Interruption of the recirculation may be caused by mutation in the connexin 26 (Cx26) and connexin 30 (Cx30) genes, which both connexins are functioned as epithelial cell gap junction system.

1.7 Gap junction protein genes

Autosomal recessive non-syndromic hearing loss make up about 80% of hereditary hearing loss (Avraham, 2001). The *DFNB1* locus, which is located on chromosome 13q11-12, was the first deafness recessive locus to be discovered (Avraham, 2001). The locus contains two genes which are *GJB2* and *GJB6* (**Figure 1.7**). The human connexins are classified by their molecular mass and by extent of sequence identity, which is indicated in the gene symbols for *GJA*, *B* and *C* subtypes. The connexin genes are very similar and contain their coding region within a single exon, separated from 5'-untranslated region (UTR) by an intron (Schrijver, 2004).

1.7.1 Gap junction protein beta-2 (*GJB2*) gene

Gap junction protein beta-2 or *GJB2* gene (Accession no.: M86849) encodes protein connexin 26 (Cx26), a gap junction protein of the beta group with a moleculer weight of 26 kDa. The size of *GJB2* gene is 2.2 kb with 2 exons and 1 intron. The coding sequence of *GJB2* encompassed entirely by exon 2 and consists of 680 bp, which are translated into a protein with 226 amino acids including the stop codon (Kenneson *et al.*, 2002) (**Figure 1.8**). Exon 1 is contained in the 5'-UTR (del Castillo *et al*., 2003).

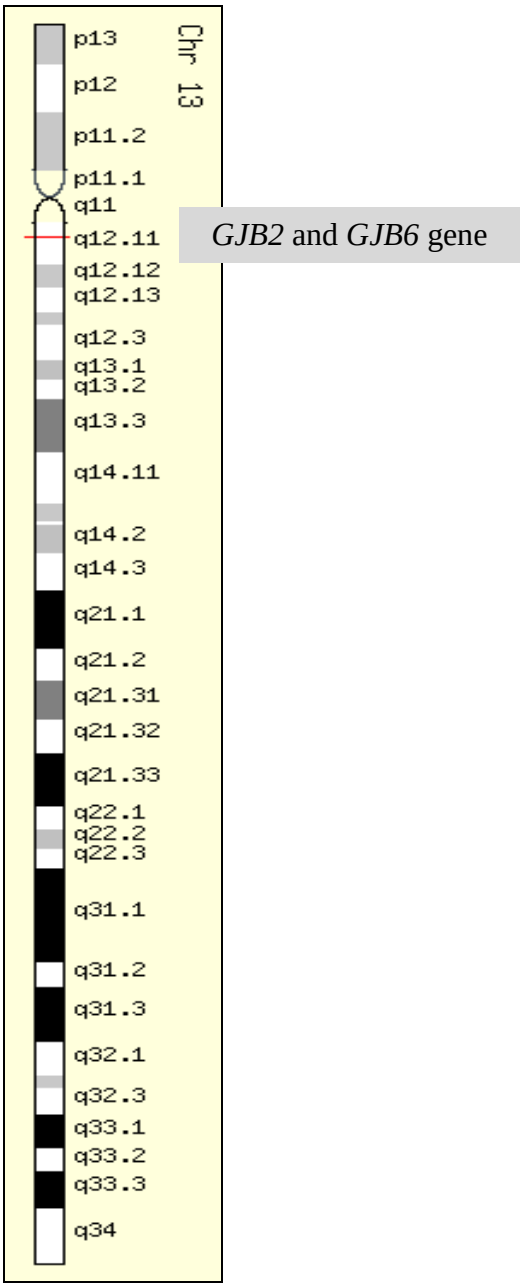

Figure 1.7: Location of *GJB2* and *GJB6* genes. Location of *GJB2* gene; at long (q) arm of chromosome 13 between positions 11 and 12 and location of *GJB6* gene; at long (q) arm of chromosome 13 at position 12 (http://ghr.nlm.nih.gov/gene=gjb2 and http://ghr.nlm.nih.gov/gene=gjb6).

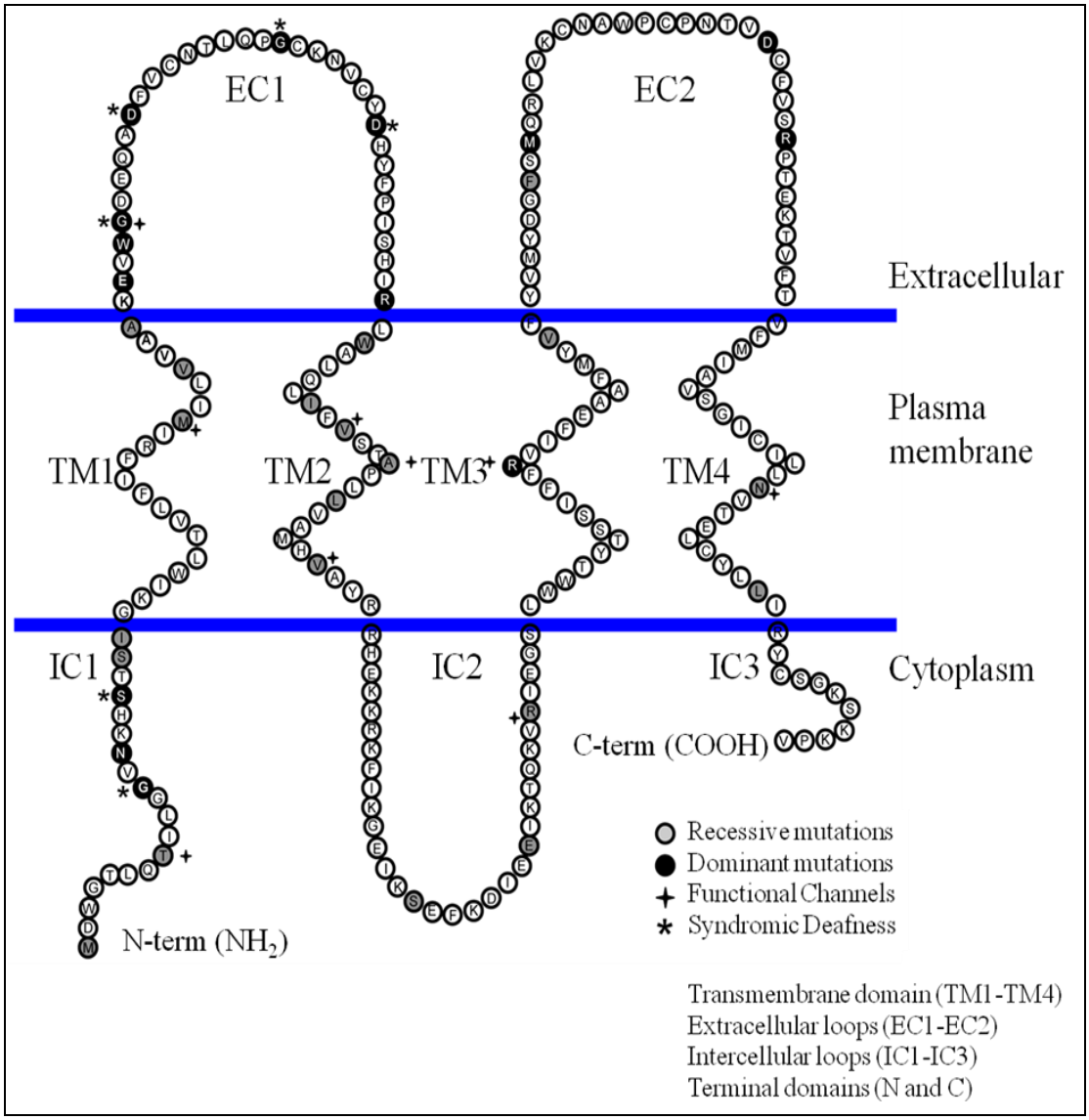

Figure 1.8: Schematic diagram of Cx26 with sequence of amino acids. Adapted from Martinez *et al*., 2009 with modification