UNIVERSITY OF NAPLES FEDERICO II

XXV Cycle
PhD Program in Neuroscience
School of Molecular Medicine

SCREENING OF DEAFNESS SUBJECTS: MOLECULAR AND FUNCTIONAL CHARACTERIZATION

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SUMMARY

1.BACKGROUND

1.1.1. GAP JUNCTIONS

Cellular communication is important for the maintenance of tissue/organ homeostasis in multicellular organisms. Using this communication, cells can review differences in environmental conditions and respond accordingly. This concept could involve either sending a signal to neighboring cells to generate a coordinated response or isolating groups of cells from the rest of the community to maintain tissue integrity (Mese et al. 2007). Contact-independent signaling is ideal for long-range communication, while contact-dependent signaling is best suited for spatially localized rapid communication (Downward 2001). Gap junction intercellular communication(GJIC) represents an important class of contact-dependent signaling. In vertebrates, gap junctions are made up of a multi-gene family called Connexins (Cxs) (Willecke et al. 2002), whereas invertebrate gap junctions are encoded by a separate gene family known as the innexins (Phelan and Starich 2001). Connexins and innexins share an identical membrane topology, but little sequence homology (Wei et al. 2004). Gap junctions (GJs) are intercellular membrane channels found in a different array of organisms spanning from nematodes, echinoderms, ascidians, mollusk, and arthropods to vertebrates such as frog, chick, rodents, and humans. Gap junctions are specialized regions of the plasma membrane in which hexameric oligomers, called connexons, dock end-to-end non-covalently across the narrow extracellular gap (figure1). These intercellular channels exclude the extracellular environment and allow the exchange of nutrients, metabolites, ions and small

molecules of up to ~1000 Da (Loewenstein 1981). GJs composed of different Cxs can have different permeabilities to molecules including AMP, ADP and ATP and ions such as K+ (Goldberg et al. 2004). Thus, the expression of multiple Cx proteins in tissue is likely to confer different properties for GJ in different cell types. The opening and permeability of GJs may be regulated by factors such as phosphorylation and dephosphorylation events, voltage, pH and chemicals.

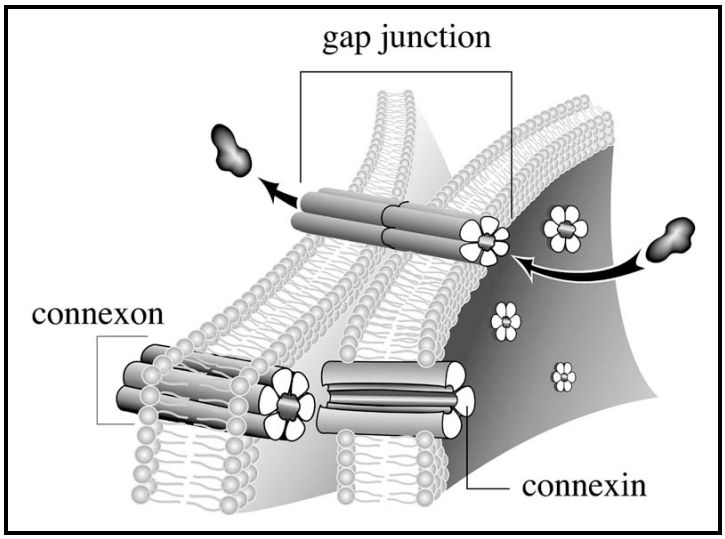

Figure1.Connexins joint to form connexon and connexons joint to form gap junction

1.1.2. THE CONNEXINS PROTEIN FAMILY

More than 20 unique connexins have been identified to date in virtually all multicellular organisms, from mesozoa to humans (Bruzzone et al. 1996; Gerido and White 2004), including a putative invertebrate connexin (Phelan et al. 1998; White 2003). In mammals connexin genes are named based on their molecular weight in Kilodaltons (KDa) (Salameh and Dhein 2005). Cxs genes are classified into two groups based on their structure and sequence homology which are α- and β-Cxs (HUGO Gene Nomenclature Committee HGNC, 2007) available at http://www.genenames.org/genefamily/gj.php). Connexins are proteins that have four transmembrane domains, two extracellular and one intracellular loop, and allow the transfer of small molecules up to 1 KDa in size. Connexins are expressed in several tissues, but in many cases their effects are restricted to particular organs and mutations in a connexin can affect one organ expressing this protein, but these mutations do not affect other tissues expressing the same protein. The exact mechanism for this selectivity for organ involvement is actually unknown and it can only partially be explained by redundancy in connexin expression or by a specific regulation at the transcriptional level as an alternative splicing. Multiple Cx types are often expressed in tissues, the diversity of which is likely to confer different properties to GJs. For example, Cx26, Cx30, Cx30.3, Cx31, Cx31.1, Cx40 and Cx43 are expressed in keratinocyte (skin) cells (Wiszniewski et al. 2000; Di et

al. 2001). The loss of one Cx protein may be compensated by the function of others in specific tissues, however, the range of Cx mutations associated with disease illustrates that this is not always possible or that specific mutants may 'gain' a deleterious function in specific tissue (White 2003; Mese et al. 2007). For example, Cx26 is expressed in the cochlea, skin, lung and hepatocytes, however some specific loss of function Cx26 mutants only cause deafness, but not skin or other disease (Kelsell et al. 1997; Carrasquillo et al. 1997; Gerido and White 2004). After gene transcription, Cxs co-translationally enter the endoplasmic reticulum (ER), however Cx26 is believed to enter the organelle either co-translationally or after translation (Zhang et al. 1996). Cxs are not glycosylated but is hypothesized to be assessed by molecular chaperones and protein disulphide-isomerases residing in the ER (Laird 2006). Connexins are synthesized in the endoplasmic reticulum (ER) and oligomerize in the ER/Golgi or trans-Golgi network to form connexons (Koval 2006). Six Cx proteins oligomerize to make up a hemichannel, called connexon. This process has been reported to occur at different cellular locations depending on the Cx. Hemichannels are transported to the cell surface in vesicles, a process believed to be facilitated by microtubules (Thomas et al. 2001; Lauf et al. 2002). After insertion into the cell surface membrane, hemichannels diffuse freely or they may dock with a hemichannel from an opposing cell to form a GJ. Connexons in adjoining cells fuse through disulfide bonding to form gap junctions (GJ) (figure1) (Hormuzdi 2004). Newly formed connexons tend to move laterally towards existing GJ channels, thus forming large gap junction plaques. Depending on the cell type and the connexin

expressed, connexons can function as hemichannels, forming a direct transmembrane communication pathway, enabling the permeation of ions, but also of small metabolites such as ATP, cAMP, IP3 and glutamate. Connexons can thus act as secretory pathways and mediate a paracrine signaling. However, they generally must be tightly regulated to avoid loss of metabolites and of ionic gradients across the membrane. In fact, aberrant opening of hemichannels usually leads to cell death, and most connexons remain in closed conformation in the physiological state (Pfenniger et al. 2011).

1.1.3. Connexin 26

Several connexins are involved in human pathologies (Pfenniger et al. 2011). It is actually reported that connexins 26, 29, 30, 30.3, 31, 32, and 43 codified respectively by GJB2, GJE1, GJB6, GJB4, GJB3, GJB1 and GJA1 genes are involved in human diseases affecting cochlea and some of them (Cx26, Cx30, Cx30.3, Cx31 and Cx43) have been involved too in syndromic form of hearing loss affecting cochlea in combination with epidermis (Rouan et al. 2001; López-Bigas et al. 2002). In particular, the GJB2 gene that encodes the connexin 26 protein is responsible for the major portion of cases of recessive or sporadic hearing loss (Kelsell et al. 1997; Zelante et al. 1997) as well for the dominant and syndromic forms (Denoyelle et al. 1998; Kelsell et al. 2001; Iossa et al. 2009). Knock-out homozygous mice for Cx26 showed embryonically lethality at embryonic 11-day (ED) (Gabriel et al. 1998). These data suggested that this was due to defective glucose uptake because the embryos were smaller but did not display any other abnormalities. This contrasts to the human phenotype of GJB2 mutations leading to deafness, many of the most frequent of which lead to the introduction of a nonsense (stop) codon and thus a truncated protein. For example, c.35delG leads to a frameshift and creates a stop codon at position 30-35. The targeted disruption of mGjb2 in the inner ear of mice only and revealed that high levels of cell death occurred soon after hearing onset, at approximately day 14 in mice, (Cohen-

Salmon et al. 2002). Initially cell death occurred in the supporting cells of inner hair cells, possibly due to apoptosis in response to sound, which then spread to the cochlear epithelial cells and sensory hair cells.

1.1.4. GJB2 GENE AND CX26 STRUCTURE

The structure of the GJB2 gene, as well the structure of other gap junction genes, is simple. 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) (figure2). GJB2 gene encodes Cx26 protein, that consists of four alfa-helical transmembrane domains (TM1–TM4), two extracellular loops (EC1 and EC2), a cytoplasmic aminoterminal domain (IC1), a cytoplasmic loop (IC2) between TM2 and TM3, and a carboxy-terminal (IC3) domain (Evans and Martin 2002) (figure3). The two latter domains are characteristic of each connexin, while the membrane spanning and the extra cellular domains are highly conserved among the entire protein family (Mese et al. 2007; Goodenough et al.1996). Under physiological conditions the extra cellular portions of a connexon interact with a connexon of opposing cell in the intercellular space to complete functional active channels. The cytoplasmic loop and the N-terminus of the protein located at the cytoplasmic side of the channel pore are involved in voltage and ion gating and are thought to be essential for the regulation of channel selectivity (Terrinoni et al. 2010).

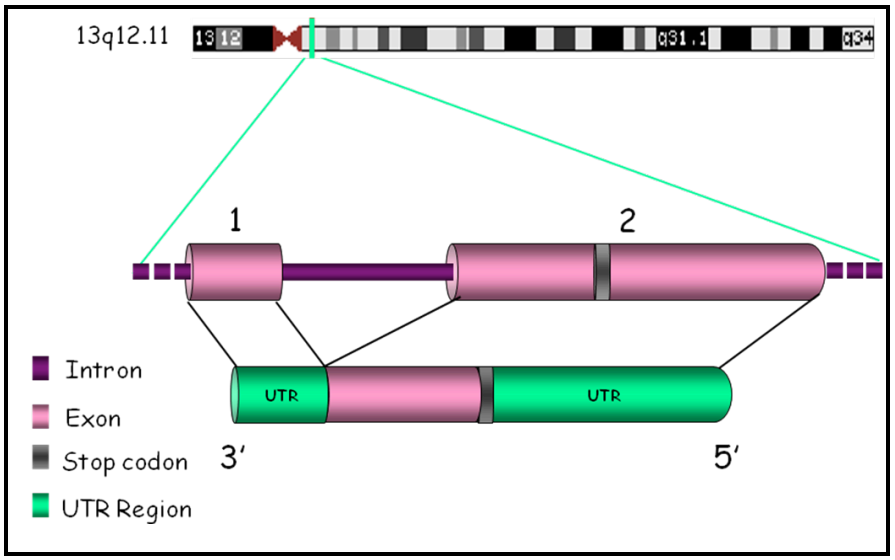

Figure2. Structure of GJB2 gene: the gene contain two exons but only encoding one to connexin26 protein

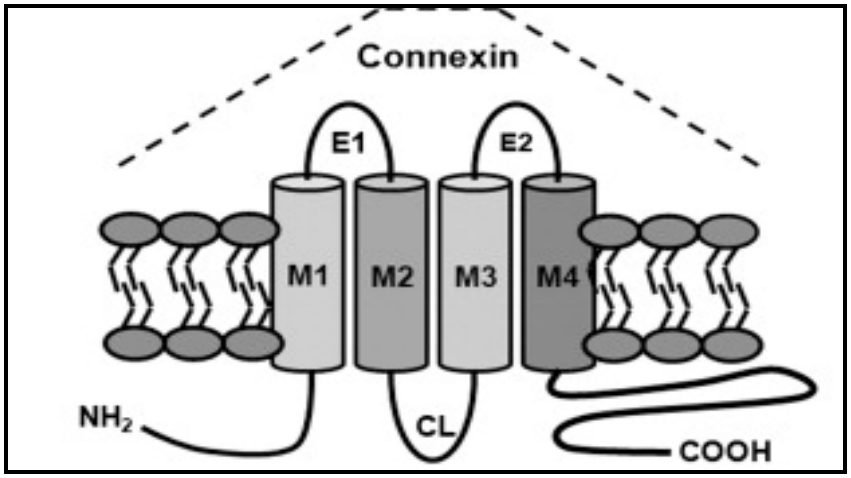

Figure3. Structure of CX26 protein in the figure are indicated the domains of the connexin26 protein

1.1.5. CX26 PROTEIN EXPRESSION

Cx26 protein is expressed in several tissues (Gerido and White 2004). In the skin the expression of Cx26 is restricted to hair follicles and sweat glands in interfollicular skin (Labarthe et al. 1998; Common et al. 2005a). Low levels are found in the basal layer and granular layer (Di et al. 2001) of palmoplantar (palm and sole) skin. Cx26 was found to be upregulated behind the wound edge after injury, implicating Cx26 in keratinocyte migration into the wound and thus in the wound healing process (Coutihno et al. 2003). In the inner ear the Cx26 protein is found in nonsensory epithelial cells surrounding the hair cells, which include supporting cells of the organ of Corti, inner sulcus cells, outer sulcus cells, and interdental cells of the spiral limbus. Cx26 is also expressed in connective tissue cells of the cochlea including strial basal cells, strial intermediate cells, mesenchymal cells lining the scala vestibuli, and fibrocytes in the spiral limbus and spiral ligament. Even if Cx26 is not expressed in hair cells, Cx26 expression in the inner ear is believed to play a major role in the formation of gap junction system.

Both dominant and recessive mutations in GJB2 are linked to Neurosensorial Hearing Loss (NSHL). All GJB2 mutations associated with syndromic diseases discovered to date are dominant and the majority of these are found in the NT (N-terminal) and ELs (Extraloops). This contrasts with NSHL mutations, which are found scattered throughout the Cx26 protein.

1.2.1. HEARING LOSS

The association of human genetic disease with specific connexin mutations including progressive neuropathy, neurosensorial hearing loss (NSHL) and hyperproliferative skin disease has established a major role for gap junctions in a different range of physiological processes (Kelsell et al. 2001; Laird 2006). Hearing impairment is a sensory disability that affects millions of people all over the world and is the most common connexin-related disease. Hearing loss comprises a broad spectrum of clinical presentation; approximately one child in 650 is born deaf (Mehl and Thomson 2002) and in many cases the cause cannot be identified and frequently it will be diagnosed as idiopathic. The genetic causes account for 50–60 % of childhood deafness. Usually, genetic deafness may be an isolated disorder, in the form of non-syndromic HL (NSHL), although frequently associated with other dysfunction. Genetic HL is present also in the form of syndromic HL (SHL), where deafness is associated with other phenotypes such as blindness (Usher Syndrome), goiter (Pendred Syndrome) or nephritis (Alport Syndrome) or keratoderma. Among the syndromic forms there are also different manifestations of mental retardation that involve deafness. During the last 15 years, about 65 of the genes responsible for hearing loss have been identified (Hereditary Hearing Loss Homepage, http://hereditaryhearingloss.org/). Several studies demonstrated that Cx26 codified by the GJB2 gene (Gap Junction Protein, Beta 2) mutations are associated with the recessive and dominant form of hearing loss (Carrasquillo et al. 1997; Kelsell et al. 1997; Zelante et al.

1997). GJB2 mutations are also found in a large proportion of cases that are apparently sporadic in origin (Estivill et al. 1998) as well as in dominant form of hearing loss (Denoyelle et al. 1998). However, the mutation spectrum in GJB2 varies among populations with different ethnic backgrounds. More than 100 mutations have been reported in GJB2 (The Connexin-deafness homepage: http://davinci.crg.es/deafness), which can be broadly divided into truncating and non-truncating mutations (Kelsell et al. 1997). No clear genotype–phenotype correlation is present but truncating mutations are generally associated with a greater degree of hearing loss than non-truncating mutations (Snoeckx et al. 2005; Cryns et al. 2004). Truncating GJB2 mutations include nonsense, splice, and frame shift mutations, and result in defective protein synthesis. The most common mutation is a deletion of a single guanine nucleotide, known as c.35delG, which results in a frameshift mutation with premature termination of protein translation (Denoyelle et al 2000; Lerer et al. 2001). Non-truncating mutations, which include deletions and insertions, and missense mutations, can affect connexin function, depending on the position and type of amino acid substitution. Recessive GJB2 mutations in regions coding for the amino-terminus, may reduce cell membrane stability (Thonnissen et al. 2002; Nickel and Forge 2008), nonfunctional or altered voltage gating gap junction (Wang et al. 2003). Mutations in noncoding regions may affect the regulation of GJB2 transcription (White 2000).

1. 2.2. NON-SYNDROMIC HEARING LOSS (NSHL) ASSOCIATED WITH GJB2 MUTATIONS

Inherited NSHL is categorized according to whether it is autosomal dominant (DFNA), autosomal recessive (DFNB) or X-linked (DFN), with 80% of cases being attributed to DFNB forms (Petit et al. 2001). Over 100 loci have been implicated in causing hearing loss (Ballana et al. 2010) however GJB2 mutations account for about 50% of prelingual genetic NSHL in Caucasians (Gasparini et al. 2000), (Rabionet et al. 2000; Petit et al. 2001; Liu et al. 2002; Pandya et al. 2003). The majority of GJB2 mutations linked to deafness are recessive. Some of the most common recessive GJB2 mutations associated with NSHL appear to affect the ability of the protein to form functional GJs, and hence are described as loss of function mutants (White 2000), (Bruzzone et al. 2003b). For example, c.35delG (also called c.30delG) results in the deletion of a guanine in a stratch of six guanines, leading to a frameshift after codon 12 and a premature stop codon, resulting in a severely truncated Cx26 protein (Zelante et al. 1997). GJs have been implicated in enabling the flow of K+ from sensory hair cells to the cochlear endolymph (Kikuchi et al. 1995) and it has been hypothesised that Cx26 mutants linked to NSHL, due to their inability to form functional GJs in the cochlea, disrupt K+ transport (Rabionet et al. 2000; Kudo et al. 2003). This appears to lead to degeneration of sensory hair cells in the inner ear and indicates that other Cxs expressed there cannot compensate for the loss of normal Cx26 function (Kudo et al. 2003). Although resulting in deafness, NSHL mutations such as c.35delG do

not cause skin disease, indicating that Cx26 expression is not mandatory in skin and that skin diseases linked to GJB2 mutations are not just due to loss of GJ function (Lee and White 2009).

1.2.3. RECESSIVE GJB2 MUTATIONS LINKED TO NSHL

Homozygosity mapping in two Tunisian consanguineous families performed by Guilford et al. (1994) demonstrated linkage of DFNB to a locus on chromosome 13q11, termed DFNB1. This region was further reduced by Gasparini et al. (1997). Kelsell et al. (1997) screened a candidate gene in this region, GJB2, and discovered the nonsense mutations in two different families with recessive NSHL which segregated with disease. More than 90 recessive GJB2 mutations associated with NSHL have been discovered to date, the full list of which can be found on the Connexin-deafness homepage (http://davinci.crg.es/deafness) (Ballana et al. 2010). Of the total GJB2 NSHL mutations reported to date, only a few of these have a high frequency in patients with NSHL (Rabionet et al. 2000). Many of the most common mutations are deletions or nonsense mutations, leading to a frameshift and a severely truncated Cx26 protein, and thus are non-functional with respect to their GJ function (Lee and White 2009).

1.2.4. DOMINANT GJB2 MUTATIONS LINKED TO NSHL

Although mutations implicated in dominantly inherited hearing impairment are not as common as those implicated in recessively inherited hearing impairment, a few DFNB1 mutations have been related to dominantly inherited hearing impairment, both non-syndromic and syndromic form. Several GJB2 mutations have been identified that can cause nonsyndromic deafness. This type of inheritance means that one copy of an altered GJB2 gene is sufficient to cause hearing loss. The altered gene instructs the incorrect replacement of a single amino acid (the building material of proteins) in the Cx26 protein. The effect of these dominant mutations remains unclear. The altered protein probably inhibits the assembly of transport channels or their normal function, which could disrupt the conversion of sound waves to nerve impulses.

1.2.5. SYNDROMIC HEARING LOSS ASSOCIATED WITH GJB2 MUTATIONS

GJB2 gene mutations have often been reported as causative for several syndromic forms of hearing loss associated to skin problems, which show all an autosomal dominant pattern of transmission. As well as for the hearing loss, the clinical phenotypes associated have a large variability and the phenotypic variability is observed even among carriers of the same connexin mutation (Heathcote et al. 2000; Uyguner et al. 2002). Syndromic HL associated with GJB2 mutations include the 'classical' form of Vohwinkel syndrome (VS), Keratitis-ichthyosis-deafness (KID) syndrome, Bart- Pumphrey syndrome (BPS), Palmoplantar keratoderma (PPK) with deafness and Hystrix-like ichthyosis-deafness (HID) syndrome (Table1) (Iossa et al. 2011). All of the known Cx26 mutations associated with syndromic disease described to date are single missense mutations, the majority of which are located in the NT or EC1 of the protein. Certain mutations can give rise to different syndromes, illustrating the heterogeneous presentation and clinical overlap between the syndromes. Functional studies have indicated that Cx26 syndromic mutants gain an aberrant function in certain tissue and have a dominant negative effect on other Cxs or on other proteins in the cell (Mese et al. 2007; Lee and White 2009). The Palmoplantar Keratoderma is a syndromic form of deafness (mild to profound). Hereditary Palmoplantar Keratoderma (PPK) comprise a clinically and genetically heterogeneous group of genodermatoses, which share impaired epidermal differentiation resulting in prominent

palmoplantar hyperkeratosis. Classically, keratoderma has been separated according to their clinical appearance into diffuse, focal, and as a feature of ectodermal dysplasias and many other syndromes (Kelsell and Stavens 1999).

Syndrome	Mutations
Keratitis-ichthyosis- deafness (KID)	G11E, G12R, N14K, N14Y, S17F, I30N, A40V, G45E, D50N, D50Y, A88V
Ichthyosis, hystrix-like-deafness (HID)	D50N, D50Y
Palmoplantar keratoderma- deafness (PPK)	Delta E42, N54H, G59A, G59R, H73R, R75Q, R75W, G130V, S183F
Vohwinkel	G59S, Y65H, D66H, G130V
Burt-Pumphrey	N54K, G59S
Unususal mucocutaneous- deafness	F142L

Table1. Syndromic forms imputable to GJB2 mutations

2. AIMS OF THE STUDY

The pathophysiological processes involved in the mechanism of auditory function are controlled by almost 100 genes and the hearing loss of genetic basis can be caused by a wide variety of mutations in different genes. So far has been identified about 65 genes responsible for various forms of hereditary deafness. Many others have not yet been identified, and for this reason at the time is not always possible, in subjects suffering, define the specific form of deafness and identify DNA alteration (mutation) responsible for the pathology. Mutations in Cx genes have been implicated in a range of diseases phenotypes. The aims of this study, through analysis of some gene responsible, is to understand the pathophysiological mechanism underlying hearing loss, to characterize by functional assays novel variations identified in the analyzed genes in order to clarify whether or not the identified variation are responsible of disease phenotype, and to effectuate a genotype-phenotype correlation in patients from Campania region with sporadic and familial deafness.

3. MATERIALS AND METHODS

3.1. CLINICAL EVALUATION

Adults patients underwent audiological evaluation by pure-tone audiometry, tympanogram, and acoustic reflex testing in both ears. To measure hearing loss severity, the air conduction pure tone average (PTA) of both ears was computed using four frequencies (500, 1,000, 2,000, and 4,000 Hz) according to the better ear. Hearing loss was categorized according to the guidelines of the European work group on Genetics of Hearing Loss: 21-40 dB as mild, 41-70 dB as moderate, 71-95 dB as severe, and more than 95 dB as profound (GENDEAF, European Thematic Network on Genetic Deafness). The clinical evaluation for children provides a diagnostic protocol, performed according to international standards (Declau et al. 2005) includes: transient evoked otoacustic emissions (TEAOE), click-evoked testing auditory brainstem response (ABR), measurement of auditory steady state responses (ASSR) and impedance test. For ABR recordings, a replicable waveform at 50 dB SPL within the expected latencies was considered “normal”. Syndromic forms of hearing loss were evaluated by specific medical control according to the alterations found such as dermatological, ophthalmology, neurological evaluation or more.

3.2. MUTATIONAL ANALYSIS

Genomic DNA was extracted by conventional salt precipitation protocols from peripheral blood samples obtained in EDTA-containing tubes or from saliva (buccal swabs) (Gentra System, DNA Isolation Kit). The presence of GJB2 mutations was ascertained through direct sequencing of amplified PCR fragments of the coding region (exon 2) of GJB2 gene. The subjects who are negative or single heterozygote for GJB2 gene mutation were analyzed for GJB3 and GJB6 genes mutation. In particular the presence of GJB3 mutations was ascertained through direct sequencing of amplified PCR fragments of the coding region of GJB3 gene; instead, for GJB6 gene, was evaluated by multiplex PCR the presence of two big deletions. Written informed consent was obtained from participants and from parents of patients younger than 18 years before blood/saliva sampling for DNA analysis.

3.3. CLONING OF THE CX26 WILD TYPE GENE

For fusion protein generation, the open reading frame of wild-type (WT) GJB2 was amplified by PCR from a genomic sequence of a wt subject using oligonucleotide primers that introduced *EcoRI* and *BamHI* restriction enzyme sites at the 5′ and 3′ termini of the cDNAs, respectively, deleting the stop codon: Cx26for: 5'-CGGAATTCAGATGGATTGGGGCACG-3' and Cx26rev: 5'-CGGGATCCACTGGCTTTTTTGACTT-3'. The resulting PCR products were digested with the two enzymes and ligated into the *EcoRI–BamHI* sites of pEYFP-N1 (Clontech, Palo Alto, CA) (figure4) followed by transformation into *Escherichia coli* (TOP10). Miniplasmid preparation and restriction enzyme analysis were performed to identify positive clones. All clones were sequenced as recommended by the manufacturer, to verify that PCR amplification did not introduce unwanted mutations.

3.4. CLONING OF THE CX26 MUTANT p.G130V

Cx26 mutation (p.G130V) was introduced into the wild-type cDNA of Cx26 using a site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene) using the same primers that introduced *EcoRI* and *BamHI* restriction enzyme sites (see above). The resulting PCR products were digested with the two enzymes and ligated into the *EcoRI* –*BamHI* sites of pEYFP-N1 (Clontech, Palo Alto, CA) (figure4) followed by transformation into *E. coli*. Miniplasmid preparation, restriction enzyme analysis and sequencing (Abi Prism 3100) were performed to identify clones harboring the mutation.

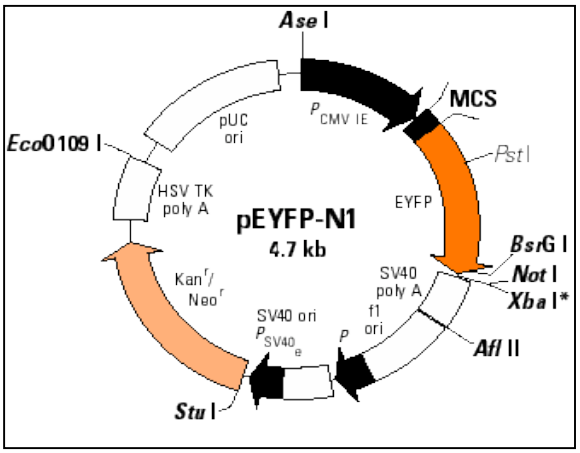

Figure4. The map of p.EYFP vector in which it was inserted the GJB2 gene

3.5. CELL CULTURE AND TRANSFECTION

The human HeLa cell line devoid of Cxs has been widely used for GJ functional studies. HeLa cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 1000 mg glucose/L, NaHCO3 and pyridoxine-HCl supplemented with 10% Fetal Bovine Serum (Thermo Scientific HyClone), L-Glutamine and 1% Penicillin 10.000U/mL-Streptomycin 10mg/mL (Sigma Aldrich). HeLa cells were incubated at 37°C according to standard procedures. At 24 h after plating on cover glass (25mm sq), cells grew to 50–60% confluence and were transfected with Cx26 constructs using the lipofectamine transfection protocol (Invitrogen) according to the manufacturer's instructions.

3.6. CONFOCAL MICROSCOPY

At 24 h after trasfection, the cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature (RT). The cells were subsequently stained for 15 minutes at room temperature using the DAPI. Fluorescence was assessed by confocal microscopy.

3.7. MOLECULAR CHARACTERIZATION OF A SYNDROMIC FAMILY

We analyzed a family who manifests a syndromic form of hearing loss with mental retardation and locomotor problems. To characterize the molecular aspects underlying this phenotype were, first of all, excluded some genes (GJB1, GJB2, GJB6 and PRPS1) as responsible of the pathology, by PCR amplification and direct sequencing. Moreover in this family was carried out a CGH-array technique to obtain more molecular information.

4. RESULTS

As reported in literature, the carrier rate of certain recessive GJB2 NSHL mutants worldwide is relatively high at 1-3% with different prevalence in specific ethnic groups, for example, c.35delG has a high frequency in European countries (Denoyelle et al. 1997; Gasparini et al. 2000; Meyer et al. 2002; Common et al. 2004).

During this work we analyzed 806 subjected affected by hearing loss for GJB2 mutations. Among affected subjects, 23% (187/806) carried mutations in GJB2 gene. In particular we identified 70/187 subjects c.35delG homozygotes, 33/187 subjects c.35delG heterozygotes, 35/187 subjects compound heterozygotes c.35delG/other, 11/187 subjects compound heterozygotes with both mutations non c.35delG and 38/187 heterozygotes subjects with other mutation different to c.35delG (figure4). Thus, we obtained a clear etiological diagnosis for 14% (116/806) of the subjects for which we can say that hearing loss is caused by genetic factors. Moreover, different new mutations [G4V (c.11G>T), D159S (c.478G>A), M195T (c.584T>C)] were identified during this study which have not yet been characterized from a functional point of view. Interestingly our study revealed a family carrying a mutation described only one times until now (Snoeckx 2005), the p.G130V (c.389G > T) mutation in the GJB2 gene. In this family both NSHL and Palmoplantar Keratoderma (PPK) are present. The mutational analysis in the GJB2 gene, revealed in the propositus (III-1) (figure5) the presence of the mutation c.35delG in compound heterozygosis with the mutation c.389G>T. The mutation p.G130V is heterozygous in the propositus' affected mother (II-1) and grandfather (I-1), indicating an autosomal dominant transmission. The unaffected father (II-2) carries the c.35delG mutation in the heterozygous

state. We realized functional studies to identified whether p.G130V mutation alter connexin trafficking and gap junctions formation. When HeLa cells were transiently transfected with Cx26-GFP wt, intercellular gap junction plaques were detected at points of cell-to-cell contact with limited punctate staining near the nuclei (figure6). Moreover, in HeLa cells transiently transfected with pG130V-GFP, also the mutant fusion protein had a predominantly membrane localization, (figure7) demonstrating that the mutation do not alter protein trafficking and gap junction formation.

In our work we have also analyzed a family affected by a form of mental retardation with deafness. Before arriving at the Unit of Audiology this family had already been subjected to some investigation as metabolic analysis, molecular analysis for X-fragile (FRAX-A) and caryotype analyses. The genealogic tree evaluation suggested a genetic condition with an X-linked transmission (figure8). The proband (subject IV-1), analyzed at five months of age, presented a severe form of bilateral neurosensorial hearing impairment. Molecular screening for GJB2 gene mutations resulted negative. The subjects was also screened for the presence of the deletions upstream the GJB6 gene involved in digenic forms of hearing loss with GJB2 (Del Castillo, 2005). Successively, at about two years of age, the proband (subject IV-1) presented several sign of neuropsicomotor delay with some peculiar characteristics of the face (broad forehead, palpebral fissure turned down, wide ears). The younger brother too (subject IV-2) and the other affected subjects (III-3, III-V) presented similar characteristics of hearing impairment and neuropsicomotor delay but, with a lesser seriousness. Peculiar characteristics of the face are absent. In the affected members, metabolic analysis, molecular analysis for X-fragile (FRAX-A)

and caryotype analyses resulted normal. The CGH-array technique revealed a big deletion of about 5Mb on the X-chromosome in the region spanning between Xq21.1- Xq31.1 containing the POU3F4 gene involved in hearing loss. Moreover, to obtain information useful for a clear genotype-phenotype correlation, all the affected subjects and the unaffected carriers will be analyzed by the molecular point of view as well as by the clinical point of view

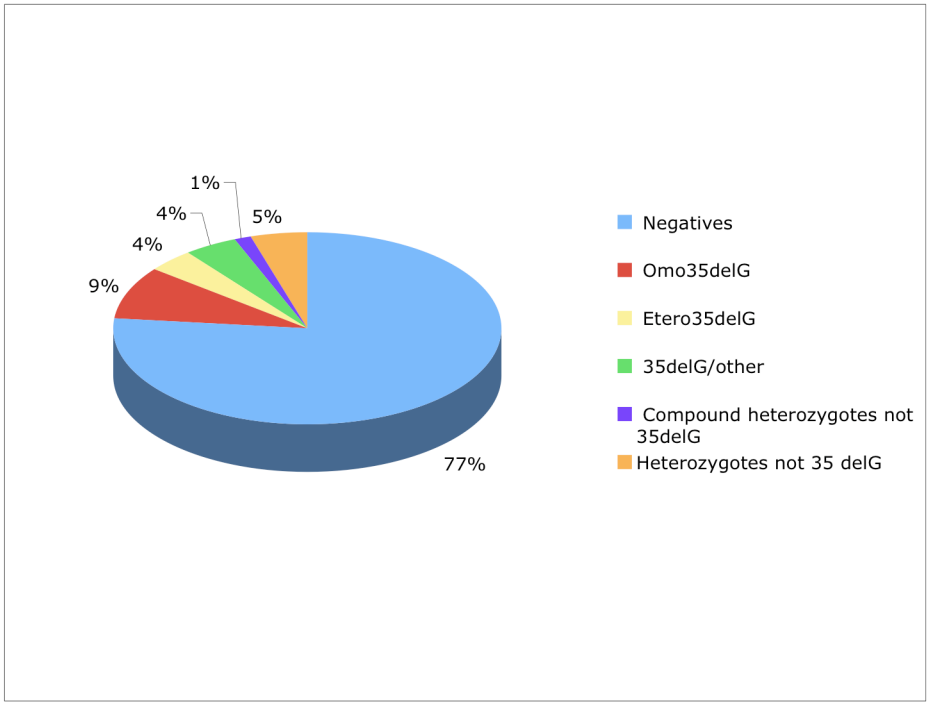

Figure 4. Percentage of affected subjects with mutations in the GJB2 gene

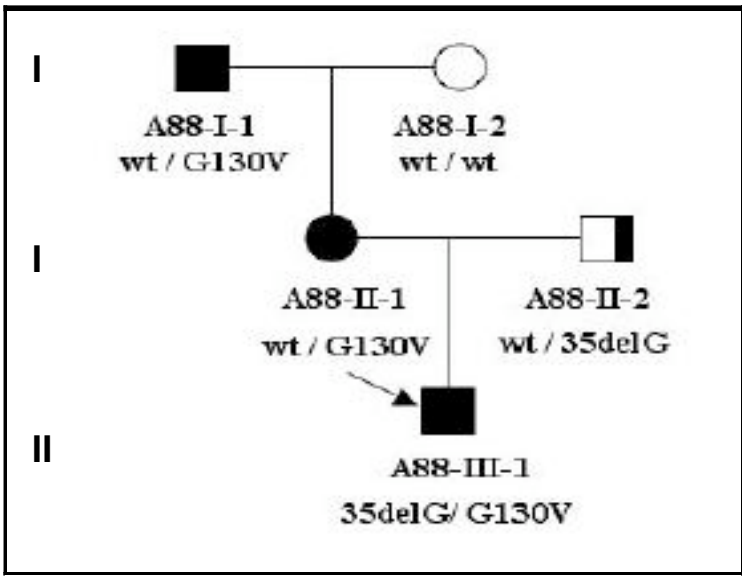

Figure 5. Pedigree of the family carrying the G130V mutation: the propositus is indicated by an arrow. Blackened symbol denote affected individuals by hearing loss

.

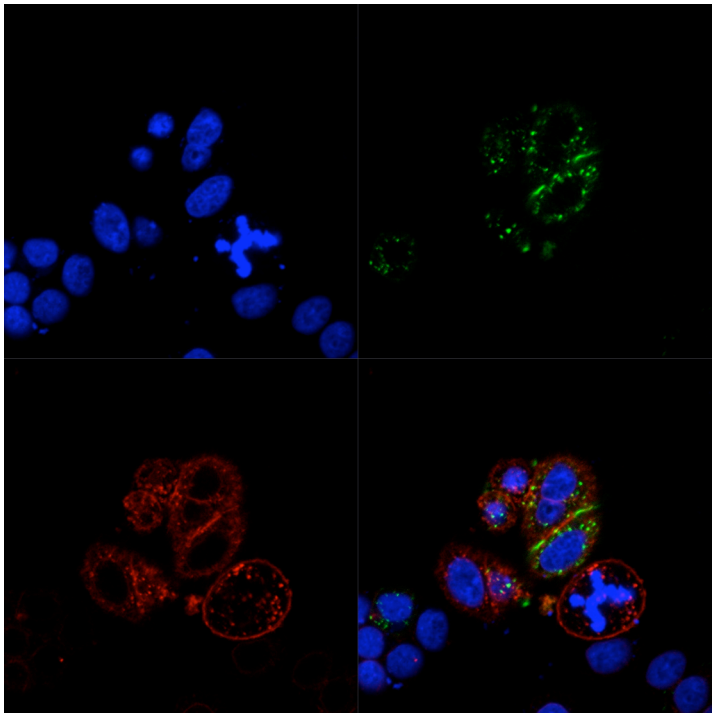

Figure 6. Localisation of wild-type-Cx26 tagged to EYFP vector transfected in HeLa cells. Bright aggregates indicative of gap junction plaques, are localized among the cells.

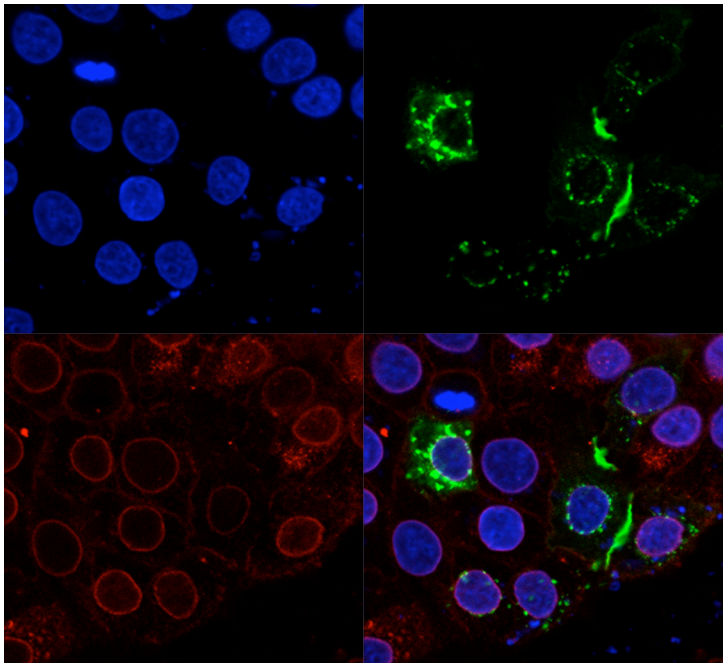

Figure 7. Localisation of G130V-Cx26 tagged to EYFP vector transfected in HeLa cells. Bright aggregates indicative of gap junction plaques, are localized among the cells.

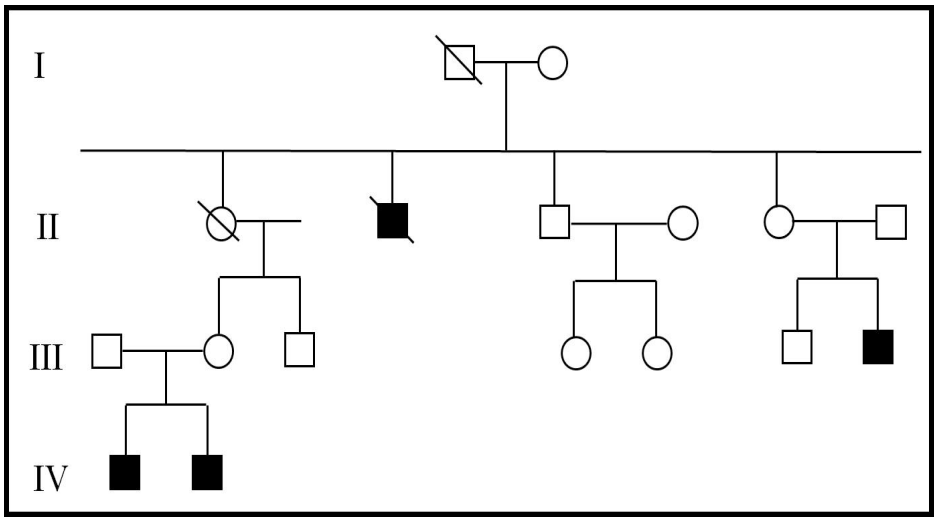

Figure 8. Pedigree of the family carrying syndromic form of hearing loss with mental retardation: the propositus is indicated by an arrow. Blackened symbol. denote individuals affected

5. DISCUSSION AND CONCLUSION

In this study, the contribution of GJB2, and other investigated genes to sensorineural hearing loss in 806 SNHL individuals, essentially from Campania region (Southern Italy), was determined. In particular by using a molecular screening for GJB2, GJB6 and GJB3 in Campania region affected subjects, a genetic etiology has been reached for the 14% (116/806) of analyzed subject. From a point of view of genotype-phenotype correlation, we can say that the individuals who have the mutation c.35delG in homozygosis, have a more severe phenotype(figure9) than 35delG/other double heterozygous or homozygous for other mutations(figure10). Moreover, the molecular screening has allowed to reveal an interesting family in which both NSHL and Palmoplantar Keratoderma (PPK) are present. This family carries p.G130V mutation, confirming the involvement of the mutation in skin abnormalities but with different expressivity. In fact the p.G130V mutation in GJB2 gene was described by Snoeckx et al, in a family affected by Vohwinkel Syndrome, an autosomal dominant genodermatosis characterized by three clinical findings: diffuse honeycomb-like keratotic thickening of the palms and soles, starfish-shaped keratotic plaques on the dorsal hands and feet, and fibrous constricting bands (pseudoainhum) at the interphalangeal joints of the hands and feet, which may result in autoamputation. In our family typical symptoms of the Vohwinkel Syndrome are not present but the three individuals carrying the mutation are all affected by hearing loss, but none of the dermatological characteristic signs of Vohwinkel Syndrome are present. Two subjects suffer only from a mild Palmoplantar Keratoderma. The peculiarity of the p. G130V mutation is its localization

in the second intracellular domain (IC2), whereas most of dominant mutations involved in syndromic form of hearing loss are localized in the first extracellular domain (EC1) of the Cx26 protein. This mutation seems originate two different clinical manifestation (PPK and VS) as observed in two different families. Until now, for this mutation there aren't functional studies. The functional studies described in this thesis try to evaluate the pathophysiological role of p.G130V mutation and revealed that there are no alteration in the protein trafficking or gap junction formation. Actually, we have not yet information on ionic current flow, but experiments through dual patch-clamp to evaluate the electrophysiological data are in progress. Moreover, to verify cytoplamic membrane co-localization of wild type and mutant proteins we are cloning the wt connexin protein in a vector expressing (pDSred-vector) the fusion protein in a different color from the previous (pEYFP-vector) we used. This construct will be co-transfected with the previous described in order to evaluate whether the mutated protein is able to form mixed connexons with the wt protein and we would like to realize an electrophysiological study in order to assess whether the mutation alters the ionic flow. In the future, we would to perform functional studies on the new other variations too, identified by us. As regards the family affected by mental retardation and deafness, several studies suggest that Mental Retardation X-Linked (XLMR) is a very heterogeneous condition and to date more XLMR genes have been identified. The mutations in most of these genes give rise to syndromic forms of XLMR where the developmental delay is accompanied by behavioral, somatic, metabolic or neurological disorders (Hildebrand, 2007; Rejeb, 2011).

Between neurological disorders frequently is reported sensorineural hearing impairment (Liu 2010). In the family that manifests syndromic form of hearing loss with mental retardation and locomotors problems we have identified a deletion of 5Mb in the X chromosome present also in the all affected members of the family. In the delete region there are several genes and among them are present POU3F4 gene involved in hearing loss but also ZNF711 gene involved in several forms of mental retardation. To obtain a detailed molecular results of this deletion are in progress analyzes - like to chromosome walking- to determine the exact breakpoints. Moreover, we will realize an exhaustive bioinformatics analysis to identify peculiar elements present in the region that can be causative of the high presence of deletions in the identified region.

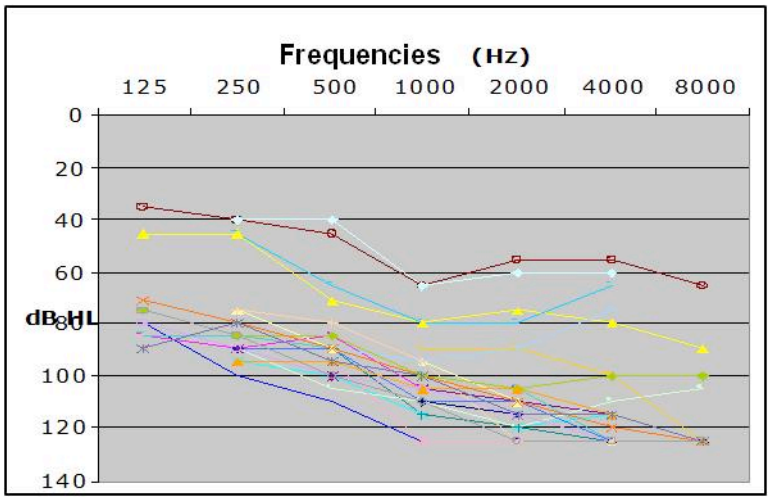

Figure9. Audiograms of c.35delG homozygotes subjects: the audiological phenotype among the maiority of subjects is present with a profound hearing deficit

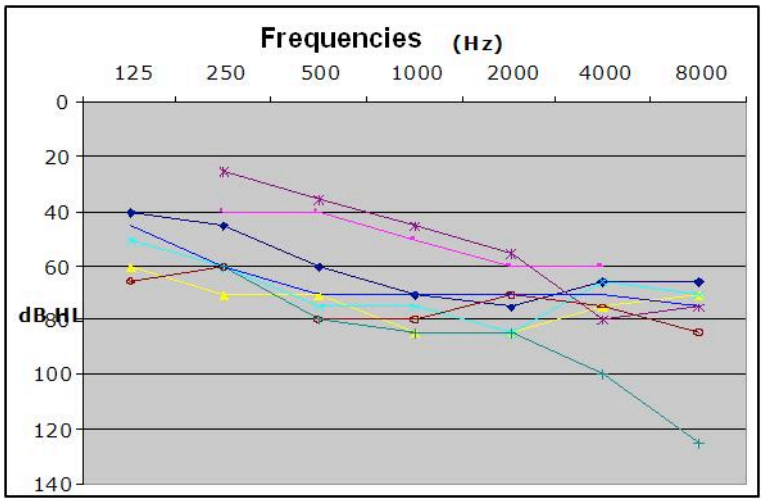

Figure10. Audiograms of double heterozygotes subjects with c.35delG: the audiological phenotype among them is less heavy respect to the c.35delG homozygotes subjects

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